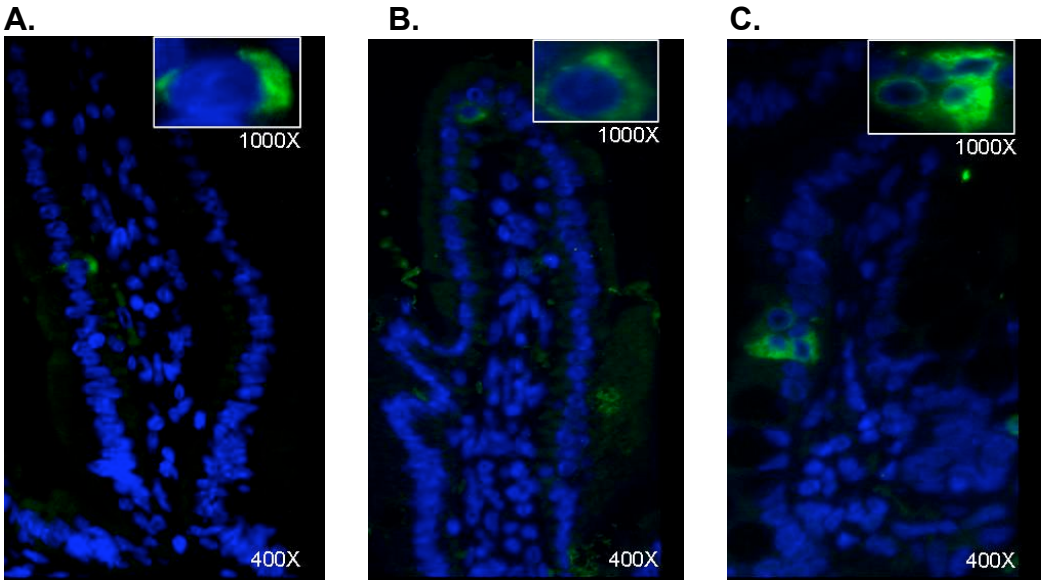
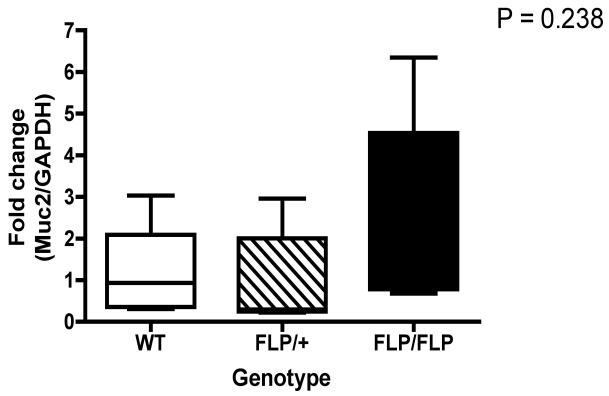
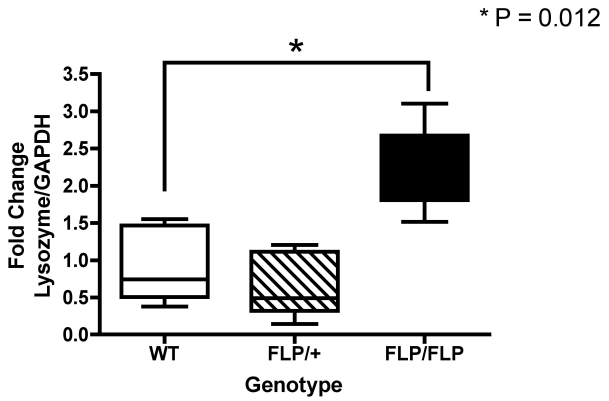


Figure 3-6. Identification of intestinal endocrine cells. Chromogranin A immunofluorescence of endocrine cells (green), DAPI counterstain of nuclei. **A.** Wild-type. **B.** ZBP-89 FLP/+ villin-cre +. **C.** ZBP-89 FLP/FLP villin-cre +.

A.



B.



C.

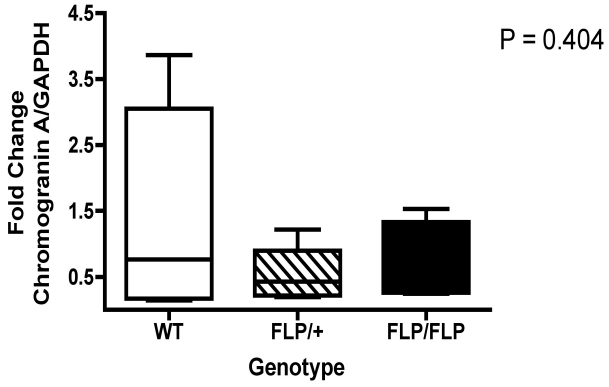


Figure 3-7. mRNA levels of intestinal secretory cells. Data shown were generated from 5 mice per genotype with each sample performed in triplicate.
A. Muc2 (goblet cell marker) expression. **B.** Lysozyme (Paneth cell marker) expression. **C.** Chromogranin A (endocrine cell marker) expression.

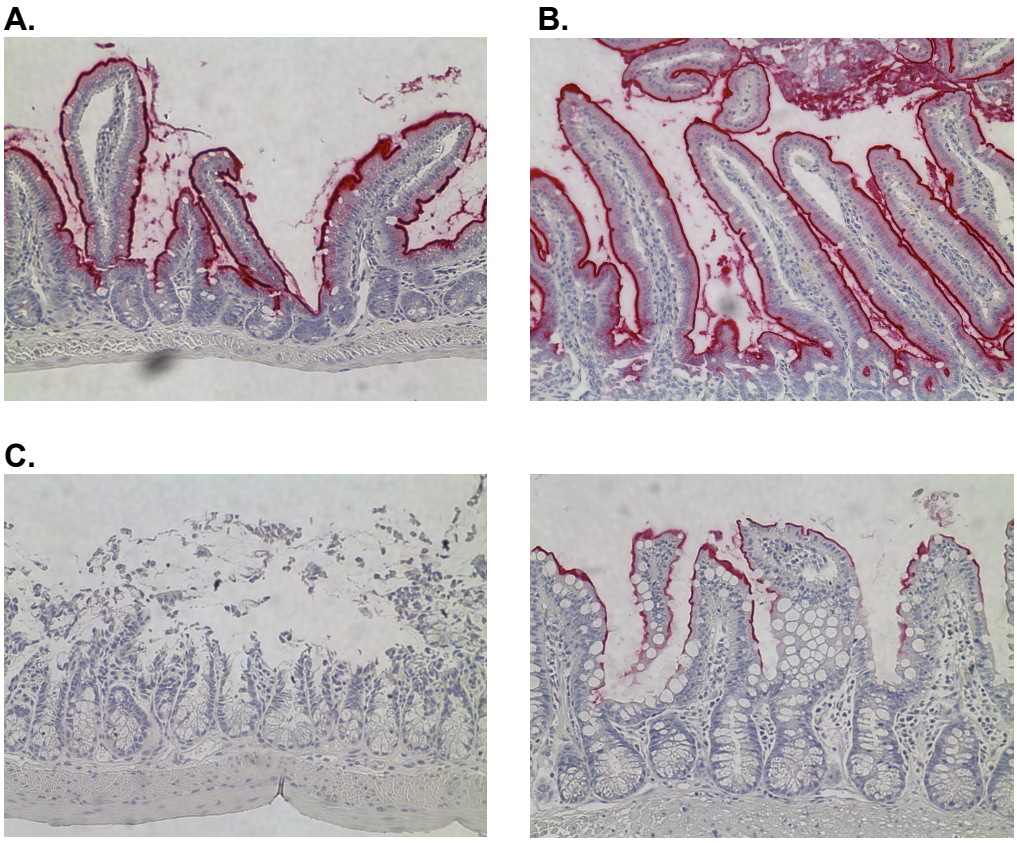


Figure 3-8. Deletion of ZBP-89 results in reduction of intestinal enterocytes. **A – C.** Alkaline Phosphatase immunohistochemistry, all images are 200X. **A.** Wild-type. **B.** ZBP-89 FLP/+ villin-cre +. **C.** 2 different ZBP-89 FLP/FLP villin-cre + mice

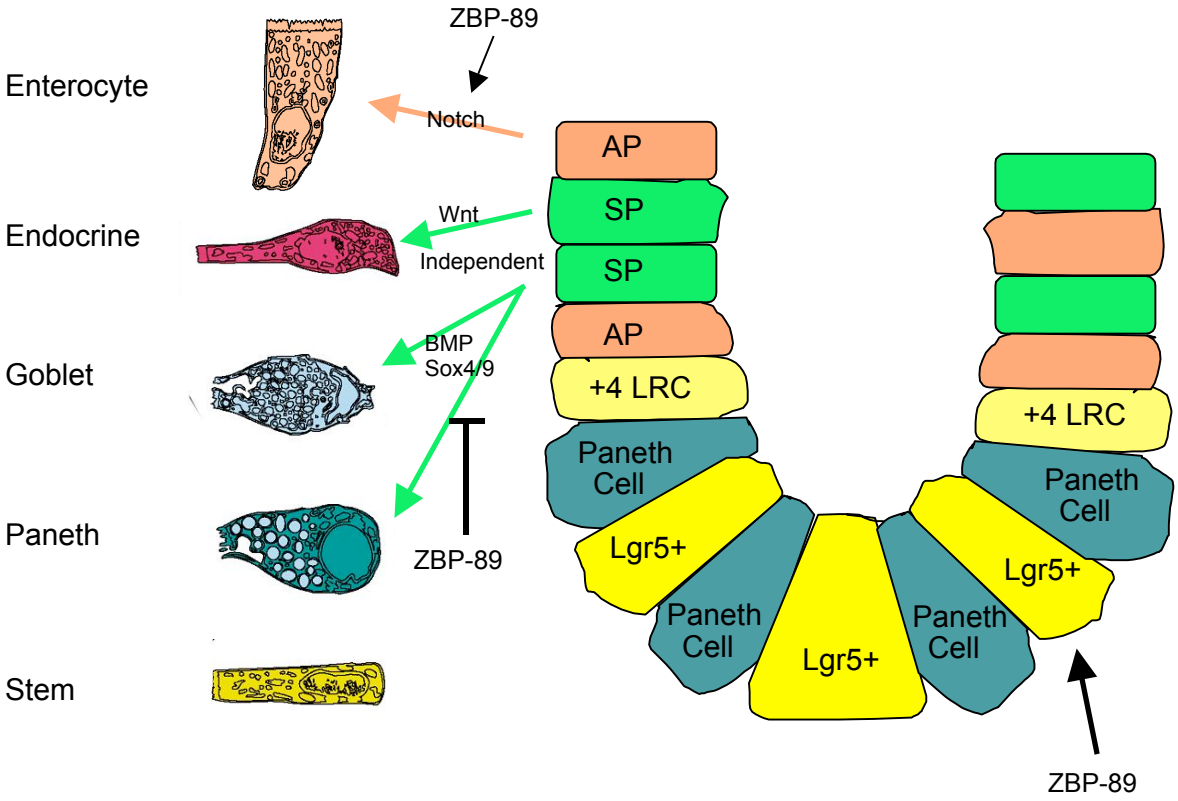


Figure 3-9. Model of ZBP-89 regulation in the small intestine. All differentiated cells arise from either Lgr5+ or +4LRC intestinal stem cells. Absorptive progenitors (AP) give rise to enterocytes through a process that requires Notch signaling. Secretory progenitors give rise to goblet and Paneth cells through Wnt/β-catenin signaling that is thought to require Sox4 and Sox9, they also produce endocrine cells through signaling processes that are independent of the Wnt pathway. Results from this study suggest ZBP-89 plays a role in the Lgr5+ stem cell, as well as in the Notch and Wnt signaling pathways.

*This figure adapted from Gastroenterology 2008;134:849-864.

References

1. **Bai, L., J. Y. Kao, D. J. Law, and J. L. Merchant. 2006. Recruitment of ataxia-telangiectasia mutated to the p21(waf1) promoter by ZBP-89 plays a role in mucosal protection. Gastroenterology 131:841-52.**
2. **Bai, L., and J. L. Merchant. 2000. Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. J Biol Chem 275:30725-33.**
3. **Bai, L., and J. L. Merchant. 2001. ZBP-89 promotes growth arrest through stabilization of p53. Mol Cell Biol 21:4670-83.**
4. **Bandres, E., R. Malumbres, E. Cubedo, B. Honorato, R. Zarate, A. Labarga, U. Gabisu, J. J. Sola, and J. Garcia-Foncillas. 2007. A gene signature of 8 genes could identify the risk of recurrence and progression in Dukes' B colon cancer patients. Oncol Rep 17:1089-94.**
5. **Barker, N., J. H. van Es, J. Kuipers, P. Kujala, M. van den Born, M. Cozijnsen, A. Haegebarth, J. Korving, H. Begthel, P. J. Peters, and H. Clevers. 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449:1003-7.**
6. **Bastide, P., C. Darido, J. Pannequin, R. Kist, S. Robine, C. Marty-Double, F. Bibeau, G. Scherer, D. Joubert, F. Hollande, P. Blache, and P. Jay. 2007. Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. J Cell Biol 178:635-48.**
7. **Bjerknes, M., and H. Cheng. 1999. Clonal analysis of mouse intestinal epithelial progenitors. Gastroenterology 116:7-14.**
8. **Bjerknes, M., and H. Cheng. 2005. Gastrointestinal stem cells. II. Intestinal stem cells. Am J Physiol Gastrointest Liver Physiol 289:G381-7.**
9. **Braunstein, E. M., X. T. Qiao, B. Madison, K. Pinson, L. Dunbar, and D. L. Gumucio. 2002. Villin: A marker for development of the epithelial pyloric border. Dev Dyn 224:90-102.**
10. **de Lau, W., N. Barker, and H. Clevers. 2007. WNT signaling in the normal intestine and colorectal cancer. Front Biosci 12:471-91.**
11. **de Santa Barbara, P., G. R. van den Brink, and D. J. Roberts. 2003. Development and differentiation of the intestinal epithelium. Cell Mol Life Sci 60:1322-32.**
12. **Djurovic, J., and M. Stevanovic. 2004. Structural and functional characterization of the human SOX14 promoter. Biochim Biophys Acta 1680:53-9.**
13. **Fevr, T., S. Robine, D. Louvard, and J. Huelsken. 2007. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Mol Cell Biol 27:7551-9.**

14. Gregorieff, A., and H. Clevers. 2005. Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev 19:877-90.
15. Grone, H. J., K. Weber, U. Helmchen, and M. Osborn. 1986. Villin--a marker of brush border differentiation and cellular origin in human renal cell carcinoma. Am J Pathol 124:294-302.
16. Hasegawa, T., A. Takeuchi, O. Miyaishi, K. Isobe, and B. de Crombrugghe. 1997. Cloning and characterization of a transcription factor that binds to the proximal promoters of the two mouse type I collagen genes. J Biol Chem 272:4915-23.
17. Hermiston, M. L., R. P. Green, and J. I. Gordon. 1993. Chimeric-transgenic mice represent a powerful tool for studying how the proliferation and differentiation programs of intestinal epithelial cell lineages are regulated. Proc Natl Acad Sci U S A 90:8866-70.
18. Humphries, A., and N. A. Wright. 2008. Colonic crypt organization and tumorigenesis. Nat Rev Cancer 8:415-24.
19. Ishizuya-Oka, A., and T. Hasebe. 2008. Sonic hedgehog and bone morphogenetic protein-4 signaling pathway involved in epithelial cell renewal along the radial axis of the intestine. Digestion 77 Suppl 1:42-7.
20. Kedinger, M., I. Duluc, C. Fritsch, O. Lorentz, M. Plateroti, and J. N. Freund. 1998. Intestinal epithelial-mesenchymal cell interactions. Ann N Y Acad Sci 859:1-17.
21. Law, D. J., E. M. Labut, R. D. Adams, and J. L. Merchant. 2006. An isoform of ZBP-89 predisposes the colon to colitis. Nucleic Acids Res 34:1342-50.
22. Law, D. J., E. M. Labut, and J. L. Merchant. 2006. Intestinal overexpression of ZNF148 suppresses ApcMin/+ neoplasia. Mamm Genome 17:999-1004.
23. Law, D. J., S. A. Tarle, and J. L. Merchant. 1998. The human ZBP-89 homolog, located at chromosome 3q21, represses gastrin gene expression. Mamm Genome 9:165-7.
24. Li, X., B. B. Madison, W. Zacharias, A. Kolterud, D. States, and D. L. Gumucio. 2007. Deconvoluting the intestine: molecular evidence for a major role of the mesenchyme in the modulation of signaling cross talk. Physiol Genomics 29:290-301.
25. Li, X., J. W. Xiong, C. S. Shelley, H. Park, and M. A. Arnaout. 2006. The transcription factor ZBP-89 controls generation of the hematopoietic lineage in zebrafish and mouse embryonic stem cells. Development 133:3641-50.
26. Lopez-Diaz, L., R. N. Jain, T. M. Keeley, K. L. VanDussen, C. S. Brunkan, D. L. Gumucio, and L. C. Samuelson. 2007. Intestinal Neurogenin 3 directs differentiation of a bipotential secretory progenitor to endocrine cell rather than goblet cell fate. Dev Biol 309:298-305.
27. Madison, B. B., K. Braunstein, E. Kuizon, K. Portman, X. T. Qiao, and D. L. Gumucio. 2005. Epithelial hedgehog signals pattern the intestinal crypt-villus axis. Development 132:279-89.
28. Madison, B. B., L. Dunbar, X. T. Qiao, K. Braunstein, E. Braunstein, and D. L. Gumucio. 2002. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J Biol Chem 277:33275-83.

29. Marshman, E., C. Booth, and C. S. Potten. 2002. The intestinal epithelial stem cell. Bioessays 24:91-8.
30. Maunoury, R., S. Robine, E. Pringault, N. Leonard, J. A. Gaillard, and D. Louvard. 1992. Developmental regulation of villin gene expression in the epithelial cell lineages of mouse digestive and urogenital tracts. Development 115:717-28.
31. Merchant, J. L. 2007. Tales from the crypts: regulatory peptides and cytokines in gastrointestinal homeostasis and disease. J Clin Invest 117:6-12.
32. Merchant, J. L., G. R. Iyer, B. R. Taylor, J. R. Kitchen, E. R. Mortensen, Z. Wang, R. J. Flintoft, J. B. Michel, and R. Bassel-Duby. 1996. ZBP-89, a Kruppel-like zinc finger protein, inhibits epidermal growth factor induction of the gastrin promoter. Mol Cell Biol 16:6644-53.
33. Moran, A., P. Iniesta, C. de Juan, C. Garcia-Aranda, A. Diaz-Lopez, and M. Benito. 2005. Impairment of stromelysin-1 transcriptional activity by promoter mutations in high microsatellite instability colorectal tumors. Cancer Res 65:3811-4.
34. Mori-Akiyama, Y., M. van den Born, J. H. van Es, S. R. Hamilton, H. P. Adams, J. Zhang, H. Clevers, and B. de Crombrugghe. 2007. SOX9 is required for the differentiation of paneth cells in the intestinal epithelium. Gastroenterology 133:539-46.
35. Passantino, R., V. Antona, G. Barbieri, P. Rubino, R. Melchionna, G. Cossu, S. Feo, and A. Giallongo. 1998. Negative regulation of beta enolase gene transcription in embryonic muscle is dependent upon a zinc finger factor that binds to the G-rich box within the muscle-specific enhancer. J Biol Chem 273:484-94.
36. Petrovic, I., N. Kovacevic-Grujicic, and M. Stevanovic. 2008. ZBP-89 and Sp3 down-regulate while NF-Y up-regulates SOX18 promoter activity in HeLa cells. Mol Biol Rep.
37. Potten, C. S., G. Owen, and D. Booth. 2002. Intestinal stem cells protect their genome by selective segregation of template DNA strands. J Cell Sci 115:2381-8.
38. Reichling, T., K. H. Goss, D. J. Carson, R. W. Holdcraft, C. Ley-Ebert, D. Witte, B. J. Aronow, and J. Groden. 2005. Transcriptional profiles of intestinal tumors in Apc(Min) mice are unique from those of embryonic intestine and identify novel gene targets dysregulated in human colorectal tumors. Cancer Res 65:166-76.
39. Robine, S., C. Huet, R. Moll, C. Sahuquillo-Merino, E. Coudrier, A. Zweibaum, and D. Louvard. 1985. Can villin be used to identify malignant and undifferentiated normal digestive epithelial cells? Proc Natl Acad Sci U S A 82:8488-92.
40. Roth, K. A., M. L. Hermiston, and J. I. Gordon. 1991. Use of transgenic mice to infer the biological properties of small intestinal stem cells and to examine the lineage relationships of their descendants. Proc Natl Acad Sci U S A 88:9407-11.
41. Scoville, D. H., T. Sato, X. C. He, and L. Li. 2008. Current view: intestinal stem cells and signaling. Gastroenterology 134:849-64.
42. Sinner, D., J. J. Kordich, J. R. Spence, R. Opoka, S. Rankin, S. C. Lin, D. Jonatan, A. M. Zorn, and J. M. Wells. 2007. Sox17 and Sox4 differentially

regulate beta-catenin/T-cell factor activity and proliferation of colon carcinoma cells. Mol Cell Biol 27:7802-15.

43. Takeuchi, A., Y. Mishina, O. Miyaishi, E. Kojima, T. Hasegawa, and K. Isobe. 2003. Heterozygosity with respect to Zfp148 causes complete loss of fetal germ cells during mouse embryogenesis. Nat Genet 33:172-6.

44. Taniuchi, T., E. R. Mortensen, A. Ferguson, J. Greenson, and J. L. Merchant. 1997. Overexpression of ZBP-89, a zinc finger DNA binding protein, in gastric cancer. Biochem Biophys Res Commun 233:154-60.

45. van Es, J. H., P. Jay, A. Gregorieff, M. E. van Gijn, S. Jonkheer, P. Hatzis, A. Thiele, M. van den Born, H. Begthel, T. Brabletz, M. M. Taketo, and H. Clevers. 2005. Wnt signalling induces maturation of Paneth cells in intestinal crypts. Nat Cell Biol 7:381-6.

46. Vidrich, A., J. M. Buzan, S. Barnes, B. K. Reuter, K. Skaar, C. Ilo, F. Cominelli, T. Pizarro, and S. M. Cohn. 2005. Altered epithelial cell lineage allocation and global expansion of the crypt epithelial stem cell population are associated with ileitis in SAMP1/YitFc mice. Am J Pathol 166:1055-67.

47. Wang, Y., M. Giel-Moloney, G. Rindi, and A. B. Leiter. 2007. Enteroendocrine precursors differentiate independently of Wnt and form serotonin expressing adenomas in response to active beta-catenin. Proc Natl Acad Sci U S A 104:11328-33.

48. Wieczorek, E., Z. Lin, E. B. Perkins, D. J. Law, J. L. Merchant, and Z. E. Zehner. 2000. The zinc finger repressor, ZBP-89, binds to the silencer element of the human vimentin gene and complexes with the transcriptional activator, Sp1. J Biol Chem 275:12879-88.

49. Woo, A. J., T. B. Moran, Y. Schindler, S. K. Choe, N. B. Langer, M. R. Sullivan, Y. Fujiwara, B. H. Paw, and A. B. Cantor. 2008. Identification of ZBP-89 as a Novel GATA-1 Associated Transcription Factor Involved in Megakaryocytic and Erythroid Development. Mol Cell Biol.

50. Yang, Q., N. A. Bermingham, M. J. Finegold, and H. Y. Zoghbi. 2001. Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science 294:2155-8.

51. Zhang, X., I. H. Diab, and Z. E. Zehner. 2003. ZBP-89 represses vimentin gene transcription by interacting with the transcriptional activator, Sp1. Nucleic Acids Res 31:2900-14.

Chapter IV

Discussion

Summary

The transcription factor ZBP-89 has roles in many vital cellular processes including gene regulation, cell growth arrest, proliferation and apoptosis (1, 2, 16, 20, 28). Understanding ZBP-89's role in these processes can have far reaching effects in health and disease states. Many studies to date have examined these roles using *in vitro* systems. While these approaches provide key clues, they do not provide an accurate perspective of the complex interplay between cell types within the whole organism. The focus of this dissertation was to target the ZBP-89 locus for deletion and to characterize the impact of this factor on the gastrointestinal tract.

Initial strategies targeted the transcription start site and p300 interaction domain of ZBP-89 for deletion (14). Instead, a functional truncated protein was generated as a result of alternate splicing and subsequent use of an alternate start site. Mice homozygous for the ZBP-89 truncated allele exhibited growth delay and increased susceptibility to DSS-induced colitis. Thus evaluation of a null locus was not possible in this case. Another published strategy targeted deletion of exons 8 and 9, which code for greater than 60% of the protein (34). Unfortunately chimeras mosaic for the heterozygous deletion resulted in reduced ZBP-89 expression and were infertile due to apoptotic death of pro-spermatogonia during embryonic

development (34). Thus analysis of ZBP-89 deletion was impeded. Moreover the latter study strongly suggested that conventional gene knock-out strategies would not generate viable animals homozygous for ZBP-89 deletion. We hypothesized that a conditional targeting strategy would produce viable mice so that deletion of ZBP-89 could be adequately studied. In Chapter II, I detail that loxP sites were placed in intron 7 and downstream of exon 9 so that when mice were crossed to those expressing Cre recombinase, the more than 60% of the coding region of ZBP-89 contained within the loxP sites were deleted by recombination. A neomycin antibiotic resistance gene was also inserted into the locus for selection of ES cell clones that have incorporated the targeting vector into their genome. This selection marker interfered with normal ZBP-89 gene function so dramatically that mice homozygous for the targeting vector experience perinatal lethality. Since the selectable marker was no longer needed after amplifying the ES clones, it was removed by crossing the mice to others ubiquitously expressing FLP recombinase enzyme. Following its removal, homozygous mice no longer experienced adverse effects and normal ZBP-89 gene and protein expression were restored. Given that the loxP sites remained in the locus, breeding the mice to any line expressing Cre recombinase will delete exons 8 and 9 in Cre expressing cells.

We chose to breed mice with those expressing Cre recombinase under control of the mouse villin promoter. This 12.4 kb promoter fragment is expressed in the epithelium of the small intestine (19). This promoter was chosen given the large body of work detailing the expression levels of ZBP-89 in colorectal cancer patients and cell lines. For example in a study analyzing gene expression in 16 patients with

Dukes' B colon cancer, both microarray analysis and real-time PCR confirmed the down-regulation of ZBP-89 (3). In a separate study examining stromelysin-1 promoter mutations in patients with high microsatellite instability (MSI) colorectal tumors, all of the mutations detected in this promoter were restricted to the region containing the ZBP-89 binding site (21). While I expected that loss of ZBP-89 would have effects on tumor formation, I also hypothesized that this loss would have greater effects on cell proliferation and differentiation in the intestine. Floxed mice experienced no viability issues within the first 2 months of age, suggesting that any effects are not serious enough to cause obvious perinatal or postnatal lethality within this time period. Upon histological analysis, it was shown that indeed perturbations to normal cell proliferation and differentiation existed in the floxed mice. Proliferation at the base of the intestinal crypts was reduced in floxed mice as measured by BrdU uptake. Goblet and Paneth cells of the secretory cell lineage were increased while enteroendocrine cells also of the secretory lineage remained unaltered. Enterocytes of the absorptive cell lineage were dramatically decreased. It is important to note that since enterocytes are responsible for nutrient absorption some functional cells must have remained otherwise the mice would not have survived. This initial characterization is very interesting and raises many possibilities to be addressed in the future when animals are in greater supply and older in age.

Future Directions

Now that we have generated a targeted ZBP-89 locus capable of being conditionally deleted, the possibilities of cell and tissue specific knock-outs are limitless. We are already collaborating with labs who will generate a null locus in the

hematopoietic cell lineage as well as smooth and skeletal muscle. Based upon its ubiquitous expression and significant mRNA in the pancreas and also T cells, it will be interesting to study its role in those tissues. Many if not all of the previously *in vitro* defined ZBP-89 functions can be examined in one capacity or another using this *in vivo* model. The next task at hand is to mate our ZBP-89 gene targeted mice with lines expressing Cre recombinase in cells and organs of interest.

The pro-apoptotic functions of ZBP-89 and its association with the tumor suppressors p300 and p53 suggest ZBP-89 may also function as a tumor suppressor. Overexpression of ZBP-89 in the intestine using the same villin promoter suppressed $Apc^{min/+}$ neoplasia (15). It would be interesting to determine if intestinal deletion of ZBP-89 would have the opposite effect and stimulate proliferation. This can be assessed using our villin-cre floxed ZBP-89 mice monitored over time for the development of tumors and/or polyps. We hypothesize that if ZBP-89 normally functions as a tumor suppressor that removal of this gene might promote tumor formation. Since tumors might take months to develop, the mice should be monitored over the course of a normal mouse lifespan for possible spontaneous tumor development. This hypothesis can also be examined through mating ZBP-89 intestinal floxed mice with $Apc^{min/+}$ mice. $Apc^{min/+}$ mice demonstrate neoplasia primarily in the small intestine and typically die between 160-180 days of age (22, 33). When mice on this background are also missing ZBP-89 they might experience earlier tumor onset compared to those solely carrying the Apc^{min} mutation. The latter study might help specifically answer the possible relationship between ZBP-89 and tumor formation more rapidly.

Another area of interest for this mouse model concerns a role for ZBP-89 in hematopoiesis. Loss of function studies in zebrafish demonstrate a requirement for ZBP-89 in megakaryopoiesis and definitive erythropoiesis (18). Specifically knock-down of ZBP-89 using morpholinos resulted in blood vessel formation but a bloodless phenotype in zebrafish embryos (18). Furthermore ZBP-89 has been shown to cooperate in a complex with GATA-1 and FOG-1 (37). Interactions between members of these two protein families are required for normal cardiac, hematopoietic, and gonadal development *in vivo* (5, 6, 24, 35). Therefore ZBP-89 might be a member of GATA/FOG transcriptional complexes required for the activation or repression of gene targets. In collaboration with the laboratory of M. Amin Arnaout we are currently studying the effects of loss of ZBP-89 in the hematopoietic cell lineage. The ZBP-89 FLP mice detailed in this thesis have been bred to mice expressing Cre recombinase under control of the MX1 promoter. This promoter is silent in normal mice but can be induced to high levels of transcription with administration of interferon alpha, interferon beta, synthetic double stranded RNA, or polyinosinic-polycytidylic acid (pIpC) either singly or in combination (7, 13, 25). This elevated expression is known to occur in bone marrow, liver, lymphocytes, and other tissues depending on the type of interferon administered. To date mice injected with pIpC on days 21, 23, and 25 after birth experience almost complete excision of ZBP-89 in bone marrow, spleen, liver, and thymus one week after the last injection (Arnaout laboratory unpublished observations). Further characterization of the loss of ZBP-89 in these tissues is currently underway. We expect that mice will experience a dramatic decrease if not total halt in blood cell

production with the excision of ZBP-89. This loss will likely be an effect of the frequency and concentration of pIpC delivered to the mice.

We would also like to breed our ZBP-89 FLP mice to a line which expresses Cre recombinase under control of a neurogenin 3 (ngn3) BAC. Ngn3 is an atonal-related bHLH protein and is required for endocrine cell differentiation in the gastrointestinal tract (10, 17, 30). Mice carrying a Ngn3 null mutation experience loss of all endocrine cells in the intestine (10). While Ngn3 is required for differentiation of these cells, it is not expressed in mature endocrine cells but rather is known to stimulate expression of pro-endocrine transcription factors such as Pax genes 4 and 6, and Neurod1 (8, 9). Ngn3 expression has also been demonstrated in goblet and Paneth cells of the secretory lineage in the intestine by mating Ngn3-cre mice to R26R mice and performing lineage tracing studies (30). It is not surprising that quantitative PCR for Ngn3 in ZBP-89$^{\Delta INT}$ mice showed slightly increased, though not significant, mRNA expression levels in homozygous mice (Figure 4-1A). Therefore upon mating ZBP-89 FLP mice to this strain, we expect to delete ZBP-89 from all intestinal endocrine cells as well as a subset of goblet and Paneth cells. Given the results from our villin-floxed-ZBP-89 mice we would expect that there would be no effect on the overall endocrine cell population however goblet and Paneth cells would likely be increased though perhaps not to as great an extent as in the study presented in this dissertation. It would be interesting to note if enterocytes were affected by this deletion, presumably ZBP-89 expression would not be deleted in these cells so we would not expect to see a difference.

While the initial characterization of our intestinal ZBP-89 floxed mice has yielded excellent results in the form of changes in cell populations much work is still to be done concerning the mechanism for such changes. While we expect that ZBP-89 is a component of signaling pathways downstream of Math1 in terms of goblet and Paneth cell proliferation this needs to be explored. Perhaps a more global role for ZBP-89 exists in the Wnt signaling pathway which can be applied to tissues outside of the intestine. Candidate interactions currently lie with the Sox genes 4 and 9, both of which are known to be key players in Wnt signaling in the intestine (4, 27, 32). While microarray analysis performed in the Merchant lab has already suggested an interaction between ZBP-89 and the Sox4 promoter might exist, this and a possible interaction with Sox9 remain to be formally confirmed. A simple co-immunoprecipitation followed by an immunoblot would quickly test this hypothesis.

An intersection between ZBP-89 and the Notch signaling pathway also remains to be explored. A current model of intestinal epithelial differentiation asserts that β-Catenin drives the production of multipotent progenitors which then use Notch signaling to select between Hes1 or Math1 proliferation markers (29, 38). Notch is a key component in the differentiation of enterocytes from absorptive progenitors, yet once again all of the factors that play a role in this signaling have not been identified (23, 31). Furthermore disruption of Notch signaling in the intestine results in increased numbers of secretory lineages and loss of enterocytes, a phenotype similar to that of our ZBP-89 intestinal floxed mice (11, 36). Our mice also demonstrate alterations in Notch-mediated lateral inhibition, which plays a role in endocrine cell patterning. Given these results a role for ZBP-89 in the Notch

pathway in the intestine is likely. Progenitors expressing Hes1 will differentiate into absorptive enterocytes, while those that express Math1 will differentiate into any of the three secretory lineages. We expect that this role lies downstream of the Notch-related transcription factor Math1, since this factor is required for all secretory lineages, however the exact placement remains to be studied. Interestingly quantitative PCR showed slightly increased levels of Math1 in homozygous floxed mice while levels of Hes1 were relatively unchanged (Figure 4-1B-C). While the exact mechanisms for our phenotype still remain at large, we have clearly identified ZBP-89 as a key player in intestinal cell proliferation and differentiation.

The hedgehog signaling, which controls patterning of the crypt-villus axis, is an additional pathway where ZBP-89 regulation may occur. Indian (Ihh) and Sonic (Shh) hedgehog are both expressed during intestinal embryogenesis at a time when cross-talk between the epithelium and mesenchyme remodel the epithelium (12). Both proteins are initially expressed throughout the intestinal epithelium but become concentrated in cells of the intervillus region due to redistribution after villus formation (26). $Ihh^{-/-}$ mice die perinatally and exhibit reduced smooth muscle as well as reduced proliferation (26). In contrast ZBP-$89^{\Delta INT}$ mice exhibit expanded smooth muscle and increased proliferation suggesting a possible interaction between these two proteins in small intestinal morphogenesis. Initial studies of Ihh expression in ZBP-$89^{\Delta INT}$ mice show no statistically significant changes, though given the small sample size and patchy Cre expression, this result does not exclude the possibility an interaction between these two proteins occurs (Figure 4-1D).

Conclusion

The goal of my dissertation was to generate mice whose ZBP-89 alleles were targeted for conditional deletion and to further delete this gene in the intestinal epithelium. Specifically, I was interested in studying perturbations in the normal cell populations of the small intestine. My findings have illustrated the complex manner in which ZBP-89 is involved in cellular proliferation and differentiation in this tissue. For example both absorptive and secretory lineages are affected by ZBP-89 loss, however not all of the secretory lineages show changes according to immunostaining and quantitative PCR. This suggests that interactions between ZBP-89 and members of the Wnt and Notch signaling pathways are likely basic helix-loop-helix (bHLH) transcription factors and/or their transcriptional targets. These findings confirm that the carboxy-terminal deletion of ZBP-89 is sufficient for disrupting its protein expression levels and provide an invaluable tool for studying ZBP-89 in this and other organ systems.

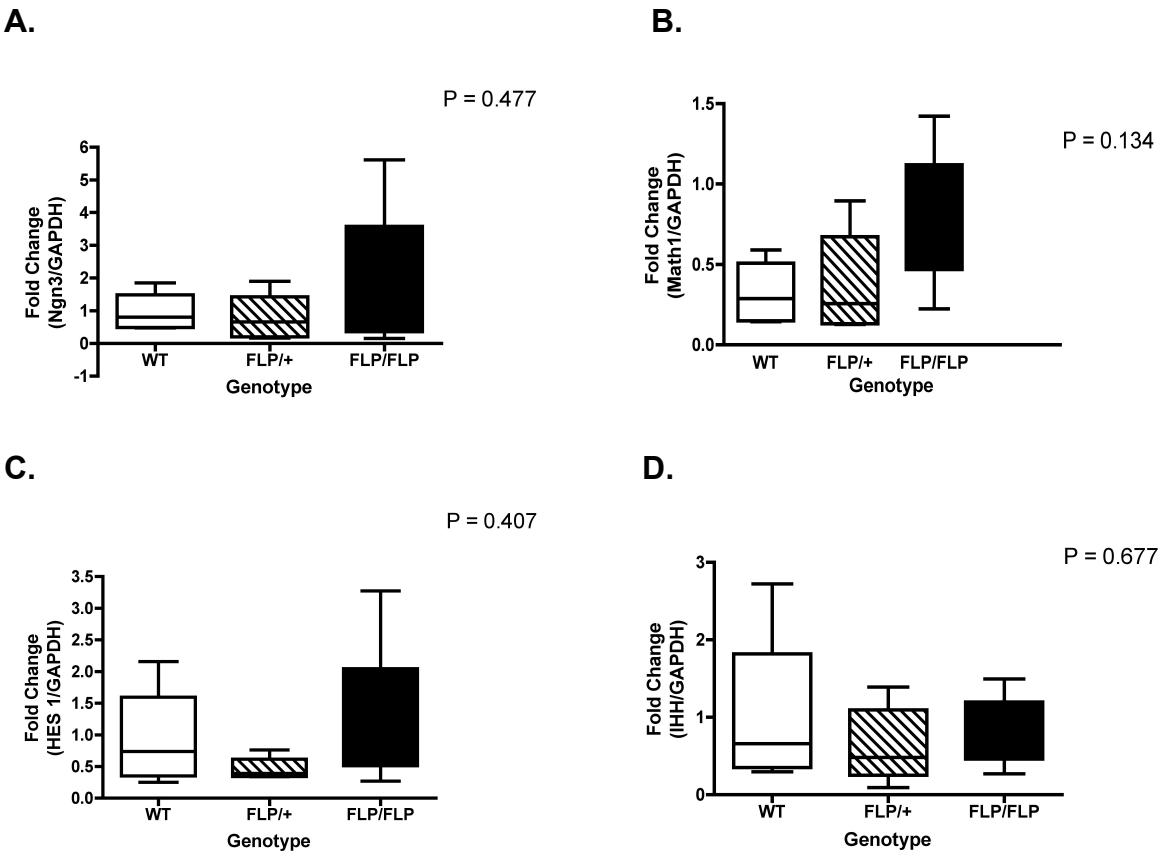


Figure 4-1. mRNA levels of hypothetical regulatory targets of ZBP-89. Data shown were generated from 5 mice per group with each sample performed in triplicate. **A.** Ngn3 (enteroendocrine cell marker) expression. **B.** Math1 (secretory lineage marker) expression. **C.** Hes1 (absorptive lineage marker) expression. **D.** Ihh (hedgehog signaling) expression.

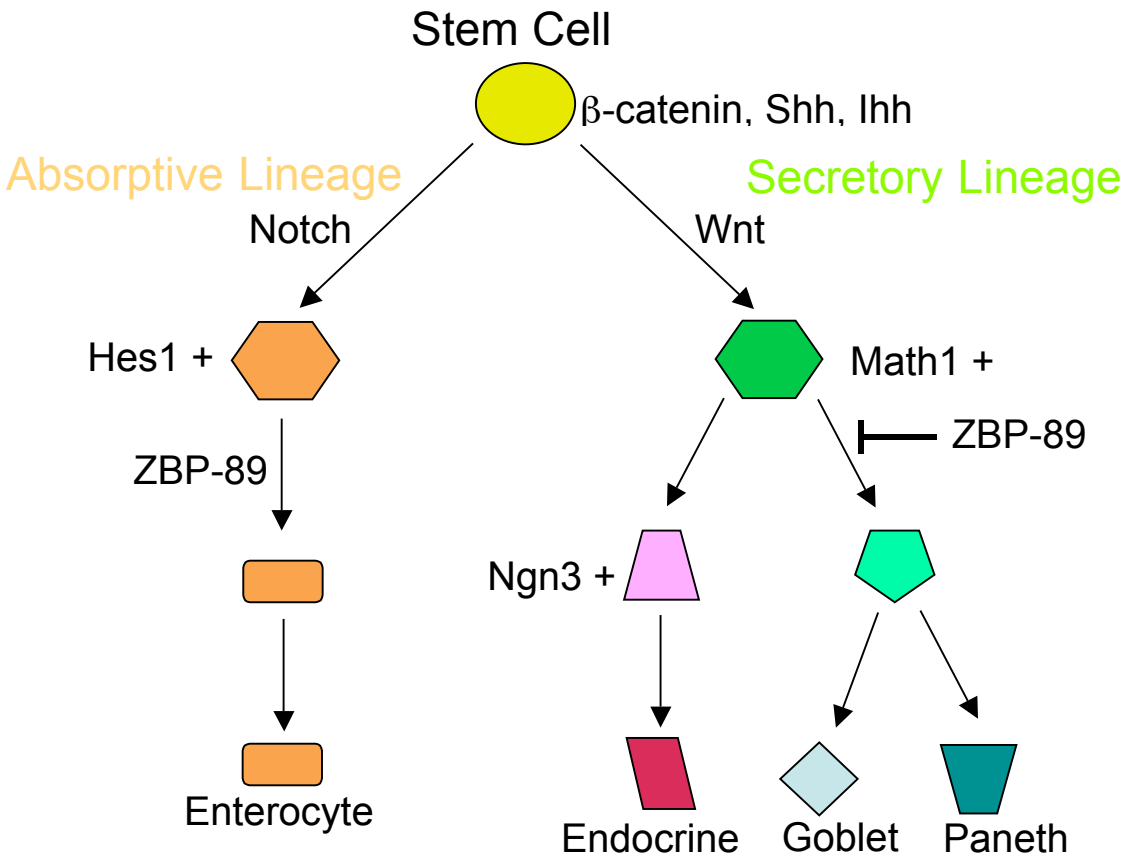


Figure 4-2. Model of ZBP-89 regulation of cellular proliferation and differentiation in the small intestine. Notch signaling is required for absorptive progenitor commitment, whereas Wnt signaling is important in driving proliferation towards secretory lineages. Current results suggest cooperation of ZBP-89 is necessary in both pathways for normal intestinal epithelial patterning.

References

1. **Bai, L., and J. L. Merchant.** 2000. Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. J Biol Chem **275:**30725-33.
2. **Bai, L., and J. L. Merchant.** 2001. ZBP-89 promotes growth arrest through stabilization of p53. Mol Cell Biol **21:**4670-83.
3. **Bandres, E., R. Malumbres, E. Cubedo, B. Honorato, R. Zarate, A. Labarga, U. Gabisu, J. J. Sola, and J. Garcia-Foncillas.** 2007. A gene signature of 8 genes could identify the risk of recurrence and progression in Dukes' B colon cancer patients. Oncol Rep **17:**1089-94.
4. **Bastide, P., C. Darido, J. Pannequin, R. Kist, S. Robine, C. Marty-Double, F. Bibeau, G. Scherer, D. Joubert, F. Hollande, P. Blache, and P. Jay.** 2007. Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. J Cell Biol **178:**635-48.
5. **Chang, A. N., A. B. Cantor, Y. Fujiwara, M. B. Lodish, S. Droho, J. D. Crispino, and S. H. Orkin.** 2002. GATA-factor dependence of the multitype zinc-finger protein FOG-1 for its essential role in megakaryopoiesis. Proc Natl Acad Sci U S A **99:**9237-42.
6. **Crispino, J. D., M. B. Lodish, B. L. Thurberg, S. H. Litovsky, T. Collins, J. D. Molkentin, and S. H. Orkin.** 2001. Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. Genes Dev **15:**839-44.
7. **Hall, M. A., D. J. Curtis, D. Metcalf, A. G. Elefanty, K. Sourris, L. Robb, J. R. Gothert, S. M. Jane, and C. G. Begley.** 2003. The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S12. Proc Natl Acad Sci U S A **100:**992-7.
8. **Heremans, Y., M. Van De Casteele, P. in't Veld, G. Gradwohl, P. Serup, O. Madsen, D. Pipeleers, and H. Heimberg.** 2002. Recapitulation of embryonic neuroendocrine differentiation in adult human pancreatic duct cells expressing neurogenin 3. J Cell Biol **159:**303-12.
9. **Huang, H. P., M. Liu, H. M. El-Hodiri, K. Chu, M. Jamrich, and M. J. Tsai.** 2000. Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. Mol Cell Biol **20:**3292-307.
10. **Jenny, M., C. Uhl, C. Roche, I. Duluc, V. Guillermin, F. Guillemot, J. Jensen, M. Kedinger, and G. Gradwohl.** 2002. Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. Embo J **21:**6338-47.
11. **Jensen, J., E. E. Pedersen, P. Galante, J. Hald, R. S. Heller, M. Ishibashi, R. Kageyama, F. Guillemot, P. Serup, and O. D. Madsen.** 2000. Control of endodermal endocrine development by Hes-1. Nat Genet **24:**36-44.

12. **Kedinger, M., I. Duluc, C. Fritsch, O. Lorentz, M. Plateroti, and J. N. Freund.** 1998. Intestinal epithelial-mesenchymal cell interactions. Ann N Y Acad Sci **859:**1-17.
13. **Kuhn, R., F. Schwenk, M. Aguet, and K. Rajewsky.** 1995. Inducible gene targeting in mice. Science **269:**1427-9.
14. **Law, D. J., E. M. Labut, R. D. Adams, and J. L. Merchant.** 2006. An isoform of ZBP-89 predisposes the colon to colitis. Nucleic Acids Res **34:**1342-50.
15. **Law, D. J., E. M. Labut, and J. L. Merchant.** 2006. Intestinal overexpression of ZNF148 suppresses ApcMin/+ neoplasia. Mamm Genome **17:**999-1004.
16. **Law, D. J., S. A. Tarle, and J. L. Merchant.** 1998. The human ZBP-89 homolog, located at chromosome 3q21, represses gastrin gene expression. Mamm Genome **9:**165-7.
17. **Lee, C. S., N. Perreault, J. E. Brestelli, and K. H. Kaestner.** 2002. Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. Genes Dev **16:**1488-97.
18. **Li, X., J. W. Xiong, C. S. Shelley, H. Park, and M. A. Arnaout.** 2006. The transcription factor ZBP-89 controls generation of the hematopoietic lineage in zebrafish and mouse embryonic stem cells. Development **133:**3641-50.
19. **Madison, B. B., L. Dunbar, X. T. Qiao, K. Braunstein, E. Braunstein, and D. L. Gumucio.** 2002. Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J Biol Chem **277:**33275-83.
20. **Merchant, J. L., G. R. Iyer, B. R. Taylor, J. R. Kitchen, E. R. Mortensen, Z. Wang, R. J. Flintoft, J. B. Michel, and R. Bassel-Duby.** 1996. ZBP-89, a Kruppel-like zinc finger protein, inhibits epidermal growth factor induction of the gastrin promoter. Mol Cell Biol **16:**6644-53.
21. **Moran, A., P. Iniesta, C. de Juan, C. Garcia-Aranda, A. Diaz-Lopez, and M. Benito.** 2005. Impairment of stromelysin-1 transcriptional activity by promoter mutations in high microsatellite instability colorectal tumors. Cancer Res **65:**3811-4.
22. **Moser, A. R., H. C. Pitot, and W. F. Dove.** 1990. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science **247:**322-4.
23. **Nakamura, T., K. Tsuchiya, and M. Watanabe.** 2007. Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. J Gastroenterol **42:**705-10.
24. **Nichols, K. E., J. D. Crispino, M. Poncz, J. G. White, S. H. Orkin, J. M. Maris, and M. J. Weiss.** 2000. Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. Nat Genet **24:**266-70.
25. **Raffel, G. D., T. Mercher, H. Shigematsu, I. R. Williams, D. E. Cullen, K. Akashi, O. A. Bernard, and D. G. Gilliland.** 2007. Ott1(Rbm15) has pleiotropic roles in hematopoietic development. Proc Natl Acad Sci U S A **104:**6001-6.

26. **Ramalho-Santos, M., D. A. Melton, and A. P. McMahon.** 2000. Hedgehog signals regulate multiple aspects of gastrointestinal development. Development **127:**2763-72.
27. **Reichling, T., K. H. Goss, D. J. Carson, R. W. Holdcraft, C. Ley-Ebert, D. Witte, B. J. Aronow, and J. Groden.** 2005. Transcriptional profiles of intestinal tumors in Apc(Min) mice are unique from those of embryonic intestine and identify novel gene targets dysregulated in human colorectal tumors. Cancer Res **65:**166-76.
28. **Remington, M. C., S. A. Tarle, B. Simon, and J. L. Merchant.** 1997. ZBP-89, a Kruppel-type zinc finger protein, inhibits cell proliferation. Biochem Biophys Res Commun **237:**230-4.
29. **Sancho, E., E. Batlle, and H. Clevers.** 2004. Signaling pathways in intestinal development and cancer. Annu Rev Cell Dev Biol **20:**695-723.
30. **Schonhoff, S. E., M. Giel-Moloney, and A. B. Leiter.** 2004. Neurogenin 3-expressing progenitor cells in the gastrointestinal tract differentiate into both endocrine and non-endocrine cell types. Dev Biol **270:**443-54.
31. **Scoville, D. H., T. Sato, X. C. He, and L. Li.** 2008. Current view: intestinal stem cells and signaling. Gastroenterology **134:**849-64.
32. **Sinner, D., J. J. Kordich, J. R. Spence, R. Opoka, S. Rankin, S. C. Lin, D. Jonatan, A. M. Zorn, and J. M. Wells.** 2007. Sox17 and Sox4 differentially regulate beta-catenin/T-cell factor activity and proliferation of colon carcinoma cells. Mol Cell Biol **27:**7802-15.
33. **Su, L. K., K. W. Kinzler, B. Vogelstein, A. C. Preisinger, A. R. Moser, C. Luongo, K. A. Gould, and W. F. Dove.** 1992. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science **256:**668-70.
34. **Takeuchi, A., Y. Mishina, O. Miyaishi, E. Kojima, T. Hasegawa, and K. Isobe.** 2003. Heterozygosity with respect to Zfp148 causes complete loss of fetal germ cells during mouse embryogenesis. Nat Genet **33:**172-6.
35. **Tevosian, S. G., K. H. Albrecht, J. D. Crispino, Y. Fujiwara, E. M. Eicher, and S. H. Orkin.** 2002. Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2. Development **129:**4627-34.
36. **van Es, J. H., M. E. van Gijn, O. Riccio, M. van den Born, M. Vooijs, H. Begthel, M. Cozijnsen, S. Robine, D. J. Winton, F. Radtke, and H. Clevers.** 2005. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature **435:**959-63.
37. **Woo, A. J., T. B. Moran, Y. Schindler, S. K. Choe, N. B. Langer, M. R. Sullivan, Y. Fujiwara, B. H. Paw, and A. B. Cantor.** 2008. Identification of ZBP-89 as a Novel GATA-1 Associated Transcription Factor Involved in Megakaryocytic and Erythroid Development. Mol Cell Biol.
38. **Yang, Q., N. A. Bermingham, M. J. Finegold, and H. Y. Zoghbi.** 2001. Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science **294:**2155-8.

Appendix

Coordinate Regulation of Opposing ZBP-89 Splice isoforms in Gastrointestinal Homeostasis

Summary

Alternative splicing enables expression of functionally diverse protein isoforms. The structural and functional complexities of the zinc-finger transcription factor ZBP-89 suggest that it may be among the class of alternatively spliced genes. We identified a human ZBP-89 splice isoform (ZBP-89$^{\Delta N}$), which lacks amino terminal residues 1-127 of the full-length protein (ZBP-89^{FL}). Although co-expressed in gastrointestinal cell lines and tissues, ZBP-89$^{\Delta N}$ mRNA was less abundant than ZBP-89^{FL}. To define its function in vivo, we generated ZBP-89$^{\Delta N}$ knock-in mice. Homozygous ZBP-89$^{\Delta N}$ mice experienced growth delay, reduced viability, and increased susceptibility to dextran sodium sulfate (DSS) colitis. The ZBP-89$^{\Delta N}$ isoform lacked the ability of full-length ZBP-89 (ZBP-89^{FL}) to repress induction of heath shock protein (HSP) 70. We conclude that ZBP-89$^{\Delta N}$ antagonized ZBP-89^{FL} function and that overexpression of the truncated isoform disrupts gastrointestinal homeostasis.

*This chapter adapted from Law DJ, Labut EM, Adams RD, Merchant JL. Nucleic Acids Res. 2006 Mar 3;34(5):1342-50. I performed RT-PCR and QPCR, as well as transfections and luciferase assays for this publication.

Introduction

Alternative pre-mRNA splicing and multiple promoter usage are common mechanisms for increasing genetic complexity in humans and mice (29, 31). Genome-wide analyses indicated that the majority of human genes express alternative splice isoforms and some variants contribute to neoplasia or other disease processes (8, 9, 25). For example, truncated isoforms of the p53 gene family, including p63 and p73, oppose the tumor suppressor activity of their full-length cognates and are over-expressed in tumors (7, 21, 28, 30).

ZBP-89 (ZNF148; Zfp148; BFCOL1), a Krüppel-type zinc-finger protein, is both structurally and functionally complex (1, 2, 6, 12, 16). It regulates diverse biological functions through direct promoter binding and through multiple protein-protein interactions (6, 16). It interacts directly with the tumor suppressor p53 through its zinc finger domain and indirectly with the histone acetyltransferase and transcriptional co-activator p300 through its amino terminus (1, 2). ZBP-89 induces cell growth arrest through a p53-dependent mechanism and apoptosis through a p53-independent mechanism (1). Mice haploinsufficient for ZBP-89 (Zfp148) are sterile due to aberrant spermatogenesis (22). In addition, embryonic stem cells harboring a single ZBP-89 allele fail to exhibit p53 phosphorylation at Ser 15 (22).

ZBP-89 forms a complex with p300 during butyrate induction of $p21^{waf1}$ in human colorectal ce3ll lines (1). The amino terminus of ZBP-89 contains an acidic domain that is required for p300 mediated induction, since an expression construct lacking this domain (Δ amino acids 1-113) loses its ability to enhance butyrate

induction of $p21^{waf1}$ (1). The acidic domain of ZBP-89 is contained entirely within exon 4 of the human and mouse genes (6). Given the heterogeneous ZBP-89 mRNA expression pattern and the functional importance of the p300 interaction domain, we therefore searched for mechanisms that could alter the expression of exon 4 (16).

We found that the ZBP-$89^{\Delta N}$ isoform, lacking the p300 interaction domain, was generated in human cells by alternative exon usage expressed from an independent promoter. ZBP-$89^{\Delta N}$ and ZBP-89^{FL} mRNAs were coordinately regulated, with the ZBP-$89^{\Delta N}$ form expressed at lower levels. To determine whether this isoform complemented or opposed full-length ZBP-89, we generated a mouse model expressing only the truncated form. Homozygous ΔNter mice experienced growth delay, reduced viability and increased susceptibility to DSS-induced colitis, suggesting that overexpression of the ΔNter isoform disrupts normal gastrointestinal homeostasis.

Materials and Methods

Organization of the human (ZNF148), chimp and mouse (Zfp148) ZBP-89 loci

A human genomic clone contig encompassing the ZBP-89 locus was previously described (12). Bacterial artificial chromosome (BAC) clones were used as templates to sequence intron/exon boundaries spanning the ZNF148 locus. Exon 4B was identified by gene prediction sequence analysis (10, 20). Similarly, a BAC and bacteriophage lambda clone contig spanning the mouse Zfp148 locus was assembled and sequenced to determine the genomic organization of the Zfp148

locus. Primers (5' to 3') Ptr-4B-F: TTC CAC CTC CCT GTC CTG TTC; Ptr-4B-R: TAT CTG TCC CGT TTG CCT G were used to amplify and sequence chimp *(Pan troglodytes)* genomic DNA containing exon 4B, after BLAST analysis had revealed a sequence gap in this region (15).

RT-PCR analysis

Total cellular RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Archived whole cell RNA samples from paired colon cancer and normal mucosal samples were also analyzed. The Titan One Tube RT-PCR System (Roche Applied Science, Indianapolis, IN) was used to generate cDNA according to manufacturer's protocols. Alternatively, 2-step RT-PCR was performed using SuperScript III RT (Invitrogen), however reverse transcription was performed at 50 C in order to obtain adequate yields of human exon 4B- containing cDNA. Primer sequences (5' to 3') were Ex2-F: GCG GAT AGA AGA GAA GAA TCA GTG G; Ex-4F: CAT TGA CGA CAA ACT GGA AGG; Ex4-R: ACT TCG ATC TTG AAG TAC TGA CTC; Ex5-R: CAG GAG AGC GTT GTT TCC G; and Ex9-R: TTG TGG CAT CTG GTG AGG. RT-PCR products were purified and subjected to DNA sequence analysis (University of Michigan DNA Sequencing Core).

Targeting vector

We constructed vector pΔEX-4 to replace ZBP-89 exon 4 with a PGK-Neo cassette by homologous recombination. High fidelity long-range PCR was used to amplify targeting arms from mouse BAC clone pBmZBP-89, using primers (5' to 3') (Kpn)Int3-F: GAT AGG TAC CGC ATT GGA TGG CAC AAG TGA CTG AGA GG;

and (Xho)Int3-R: CTC GAG CCC GGG CTT AAG TAT AAC TGC CTA GAA AG for the left arm; and (Apa)Int4-F: CTC GAG GGG CCC GTA AGT ACT AAA CTA GAA ATG; and (Apa)Ing4-R: CTC GAG GGG CCC AAG AGC CTT GCT GAC TCA TAG for the right arm. The left targeting arm, encompassing exon 3 and intron 3, was 8 kb in length. The right targeting arm consisted of the proximal 2 kb of intron 4. The neomycin cassette, including a transcriptional stop signal, was isolated from pPNT, generously provided by Dr. Richard Mulligan (24).

Generation of targeted ES cells and ΔNter mice

Electroporation of embryonic stem (ES) cells with the pΔEx-4 targeting vector and microinjection of blastocysts with targeted ES cells were performed by the University of Michigan Transgenic Animal Model Core (www.med.umich.edu/tamc). Genomic PCR was utilized to genotype targeted ES clones, chimeric founders, and progeny resulting after germline transmission. Primer sequences (5' to 3') were Int3-F1: GGA GTA TTC TCT GTC CGT TAT G; Int4-326R: GCA AGA ACT ACA CAG AGA AAC CAC; and R506Neo: TGA GGA AGA GGA GAA CAG CG. The data reported here are derived from offspring on mixed C57BL/6 and 129/Sv/J genetic backgrounds.

Western blot analysis

Whole cell protein extracts were prepared from mouse spleen and liver using T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL), supplemented with Complete Mini protease inhibitors (Roche, Indianapolis, IN) according to the

manufacturer's recommendations. SDS/PAGE, electroblot transfer to PVDF membrane and immunoblot procedures were as previously described (1, 16).

Dextran Sodium Sulfate (DSS) colitis

DSS colitis was induced in 6- to 9-month old ZBP-89$^{\Delta N}$ and control mice by the addition of 4% DSS to drinking water for a period of 5 days (18). Treated mice were returned to normal drinking water for 2 days prior to necropsy for histopathological scoring. Hematoxylin and eosin (H&E) stained colon sections were prepared by the Swiss roll method and were scored for colitis index by a modification of a previously described method, briefly as follows (14, 19). Crypt damage (0 to 3), none, basal only, moderate damage, complete erosion, respectively. Inflammation (0 to 3), none, minor, moderate and severe leukocyte infiltration. Submucosal edema (0 to 3), none, minor, moderate, severe, respectively. Hemorrhage (0 to 3), none, minor, moderate, severe. Each parameter was multiplied by an extent factor (1 to 3), <10%, up to 25%, 25 to 50%, and >50% respectively. Samples with transmural involvement received an additional 4 points, for a maximum possible colitis index of 40. Animals that died during treatment due to colitis injury were also given the maximum score. Samples were blinded and scored independently by two individuals.

Cell culture and transfections

The following cell lines were obtained from ATCC (Manassas, VA) and maintained on the recommended growth media: Human gastric epithelial cells Kato III and MKN45; human colon cancer cells Colo 320DM, CaCo-2, and HCT116; and

human Jurkat leukemia cells. Cells were cultured in a humidified atmosphere with 5% CO2 and 95% air at 37 C. All culture media were supplemented with penicillin G (100 U/mL). Cells were grown on 6-well plates and transfected using Fugene6 (Roche, Indianapolis, IN) according to the manufacturer's recommendations. Luciferase assays were performed using a Wallac luminometer (Victor 3, model 1420) as previously described (1, 2).

Expression and reporter constructs

Mouse cDNAs encoding ZBP-89 were amplified by RT-PCR from mouse liver RNA and cloned into pcDNA3.1V5-His (Invitrogen) using primers (5' to 3') 4A-For: ATG AAC ATT GAC GAC AAA CTG GAA GGA TTG WITH 794-Rev: GCC AAA AGT CTG GCC AGT TGT GGC ATC for ZBP-89^{FL}; and M128-For: ATG AGA GAC AAA AAA CAA GTC AGA with 794-Rev for ZBP-89$^{\Delta N}$. The structure of each construct was confirmed by DNA sequencing of both strands. The human inducible HSP70 reporter (SPV-130C) was obtained from Stressgen Bioreagents (Victoria, BC, Canada) and used according to the recommended procedure.

Results

To investigate the complexity of ZBP-89 expression, we screened the human ZNF148 genomic locus *in silico* for alternative exons and splicing mechanisms (10, 20). After localizing alternative exon 4B within 4 kb upstream of exon 5 (Figure 1A), we used RT-PCR analysis to show that was functional (Figure 1B). Exon 4B mRNA was expressed at lower levels relative to exon 4A-containing transcripts in gastric cancer (MKN45 and Kato III) and colon cancer cells (ColoDM2, HCT116, CaCo2,

and primary tumor). Both forms were abundantly expressed in Jurkat T-cell leukemia cells (Figure 1B). The variant transcript also was expressed as a minor component in normal colon (data not shown). This suggested that at least two isoforms interact to mediate ZBP-89 activity.

To better understand its contribution to ZBP-89 function, we sequenced exon 4B-containing cDNA. Exon 4B mRNA included exons 5 and 6 (Figure 2A), as well as exons 7-9 (data not shown). In contrast, exons 1-4A were absent from the alternative transcript (data not shown), suggesting that an independent promoter regulates exon 4B expression. The DNA sequence of cloned variant cDNA (Figure 2A) showed that exon 4B was spliced to exon 5 resulting in an alternative reading frame relative to the cDNA encoded by exon 4A (Figure 2B). Exon 4B was 329 nt in length and composed of untranslated sequences when fused to exon 5. This resulted in the utilization of an alternative initiation codon corresponding to M128 of full-length mRNA. These data predicted that alternative splicing of exon 4B resulted in the expression of an amino-terminally truncated ZBP-89 isoform ZBP-89$^{\Delta N}$. This isoform lacks the acidic domain and p300-interaction region found in full-length (ZBP-89FL) protein (1, 16). Identical results were obtained with cDNA derived from esophagus, stomach, colon and Jurkat T-cells, suggesting that the exon 4B alternative splicing mechanism is common. Although the human (ZNF148) and mouse (Zfp148) ZBP-89 loci share many features of genomic organization, exon 4B is absent in mice and most other mammals (data not shown) (6). The exception, to date, is the chimpanzee (Pan troglodytes), which shares 99% DNA sequence

identity over the 329 bp segment with homology to exon 4B (Figure 3). This suggests that the ZBP-89$^{\Delta N}$ isoform splicing mechanism is restricted to hominids.

Since mice lack exon 4B, and therefore do not express a ZBP-89 $^{\Delta N}$ isoform, we targeted mouse exon 4 to test the functional significance of the amino-terminal domain. Conventional gene targeting strategies, utilizing homologous recombination in embryonic stem (ES) cells, were used to replace exon 4 with a neomycin phosphotransferase cassette (Figure 4A). The left targeting arm contained exon 3 and intron 3, and the right targeting arm contained 1.8 kb of the 5' margin of intron 4. Homologous recombination occurred in 4 of 1200 ES clones identified by PCR (Figure 4B). Of the 4 homologous recombinants, 3 demonstrated stable karyotypes and were therefore injected into blastocysts. Germline transmission of the exon 4-targeted locus resulted in two founder lines, 4E10 and 8C6, while a third founder, 9C6, failed to transmit the recombinant locus. Germline transmission was obtained first in the 4E10 lineage. Near-Mendelian rations were observed at the age of weaning (3 wks) in offspring of heterozygous intercrosses (Figure 4C). The data suggested that about 20 percent of Δexon 4 homozygous embryos died *in utero* or perinatally, however the difference between predicted and observed ΔExon4/ΔExon4 yields was not statistically significant (Chi square analysis; $p > 0.05$). A similar pattern was observed in the 8C6 lineage.

To determine the effect of exon 4 deletion on ZBP-89 expression, we analyzed cDNA derived from normal, heterozygous, and exon 4-targeted homozygous animals (Figure 5A). Whereas exon 4 sequences were absent from

recombinant mRNA, both upstream and downstream exons were expressed. RT-PCR with primers spanning exon 4 generated a 690 bp cDNA fragment from wildtype and a 339 bp cDNA from mutant alleles. The size difference between wildtype and targeted cDNA was 351 bp, the size of exon 4. This suggested that exon 3 was spliced directly to exon 5, excluding the intervening Neo cassette, and this was confirmed by DNA sequencing (Figure 5B). Attempts to quantify ZBP-89 expression levels in the targeted locus proved unfruitful by quantitative RT-PCR. Deletion of exon 4 removed the initiation codon found in full-length message and also resulted in an alternative reading frame. Gene feature sequence analysis predicted that the alternative initiation codon corresponded to M128 of the full-length protein (Figure 5B), analogous to the human ZBP-89$^{\Delta N}$ variant (Figure 1C) (10, 20).

Western blot analysis confirmed that ZBP-89^{FL} protein was absent in recombinant mice (Figure 5C). A polyclonal antibody raised in rabbits with an antigen corresponding to amino acid residues 1-521 detects full-length ZBP-89 in wildtype and heterozygous mice due to recognition of an amino-terminal epitope (1, 16). Thus the truncated form, missing the amino-terminal epitope, could not be detected in ZBP-89$^{\Delta N}$ homozygotes. Collectively, the mRNA and protein expression data confirmed, albeit by different molecular mechanisms, that we successfully knocked into the mouse genome the ZBP-89$^{\Delta N}$ variant previously confined to hominids.

At weaning, by age 3 weeks, male ZBP-89$^{\Delta N/\Delta N}$ mice weighed an average of 5.9 +/- 0.5 g, 34% smaller that ZBP-89$^{FL/FL}$ (12.1 +/- 0.7 g) and 49% smaller than

ZBP-89$^{FL/\Delta N}$ heterozygous littermates (15.1 +/- 0.8 g) (Figure 6A). From ages 4 weeks to 8 weeks, the size differential between ZBP-89$^{\Delta N/\Delta N}$ and control mice was diminished. A similar effect was observed with female mice (data not shown). As shown above, ZBP-89$^{\Delta N/\Delta N}$ mice experienced 20% perinatal mortality as determined by genotype ratios at weaning (Figure 4C). Reduced viability persisted and became statistically significant, with 50% mortality at 48 weeks and 69% mortality by 104 weeks (Figure 6B). The 2-year survival ratios were 20 of 20 ZBP-89$^{FL/FL}$, 39 or 40 ZBP-89$^{FL/\Delta N}$ and 5 of 16 ZBP-89 $^{\Delta N/\Delta N}$ mice. Histopathological organ and tissue surveys revealed no obvious abnormalities in ZBP-89 $^{\Delta N/\Delta N}$ mice, with the possible exception of the colon, where we noted a trend toward slightly increased lymphocytic infiltrates (data not shown). Collectively, these data demonstrate that balanced expression of ZBP-89^{FL} and ZBP-89$^{\Delta N}$ isoforms, as was seen in heterozygous mice, supports normal growth and viability. In contrast, exclusive expression of the ZBP-89 $^{\Delta N}$ isoform in ZBP-89$^{\Delta N/\Delta N}$ mice results in growth delay and reduced viability.

We previously showed that the amino terminal region of ZBP-89 (amino acids 1-111) was required to potentiate butyrate induction of p21^{waf1}, suggesting that the amino terminal region mediates the butyrate-dependent anti-proliferative activities of ZBP-89 in the mammalian gastrointestinal tract (1). Butyrate suppressed colonic inflammation when induced by dextran sodium sulfate (DSS) (17). Since DSS accentuates a tendency to develop colitis, we challenged ZBP-89$^{\Delta N/\Delta N}$ mice acutely with 4% DSS for five days, as previously described (18). Histopathological analysis (hematoxylin and eosin stained sections) demonstrated the presence of severe colitis that correlated with a ZBP-89$^{\Delta N}$ gene dosage effect (Figure 7A-C). ZBP-

89$^{FL/FL}$ mice had localized areas of lymphocytic infiltration and minimal submucosal edema (Figure 7A). ZBP-89$^{FL/\Delta N}$ mice exhibited more extensive infiltration, with displaced normal crypt architecture in addition to submucosal edema (Figure 7B). ZBP-89$^{\Delta N/\Delta N}$ mice had areas with complete erosion of crypt architecture, grossly visible hemorrhage and extensive submucosal edema (Figure 7C). The ZBP-89$^{\Delta N}$ gene dosage effect also correlated with the duration of gastrointestinal bleeding (Figure 7D). Moreover, only ZBP-89$^{\Delta N/\Delta N}$ mice died during DSS treatment, with 50% mortality by the conclusion of the 7-day regimen (Figure 7E). Composite colitis scoring similarly paralleled the ZBP-89$^{\Delta N}$ gene dosage (Figure 7F). These data suggest that increased expression of ZBP-89$^{\Delta N}$ is associated with increased susceptibility to colitis.

DSS-induced colitis in rats is associated with elevated expression of inducible heat shock protein (HSP) 70 (also known as HSP72) (26). Moreover, butyrate enemas protect rats against colitis and this effect is associated with down-regulation of HSP72, suggesting that HSP72 is a mediator of DSS-induced colitis (26). Using microarray analysis of ZBP-89 targets, we previously found that ZBP-89 and HSP72 expression levels were inversely correlated (Bai and Merchant, unpublished), suggesting that FL ZBP-89 repressed HSP72 expression. To determine if HSP72 mediated the effects of ZBP-89 expression on colitis susceptibility, we compared the ability of ZBP-89^{FL} and ZBP-89$^{\Delta N}$ isoforms to regulate basal and heat-shock induced HSP72 promoter activities (Figure 8). Co-transfection of HT-29 colon cancer cells with a ZBP-89^{FL} expression vector and the HSP70.2 promoter reporter resulted in a 44% reduction in heat shock-inducible promoter activity, relative to the empty vector

control. The ZBP-89$^{\Delta N}$ expression construct had no significant effect on heat shock induction of the HSP72 promoter, thus demonstrating that the ΔN isoform lacks the ability of FL protein to inhibit stress-induction of HSP72. There was no difference seen in basal HSP72 levels. These data suggest that failure of the ZBP-89$^{\Delta N}$ isoform to inhibit HSP72 promoter induction may account for increased susceptibility of ZBP-89 ΔN/ΔN mice to DSS-induced colitis.

Discussion

Through structural and functional analyses, we identified a human-specific ZBP-89 splice isoform, ZBP-89$^{\Delta N}$, which was generated by alternative promoter usage and alternative splicing. As a consequence, ZBP-89$^{\Delta N}$ protein lacks a transcriptional domain found in its full-length ZBP-89 cognate. This is the first characterization of a ZBP-89 isoform and is consistent with previous observations that gene complexity is lower in mice than in humans. As many as 59% of human genes are alternatively spliced, while the highest estimate to date for the mouse is 33% (3, 11). Emerging genomic sequence data suggests that the ZBP-89 $^{\Delta N}$ splicing mechanism has been conserved between humans and *Pan troglodytes*, indicating that the mechanism of regulating ZBP-89 function is restricted to hominids.

Alternative mRNA splicing increases genetic diversity through regulatory mechanisms that also are implicated in cancer and other disease processes (4, 5, 8, 9, 25, 27, 29). The human ZBP-89 ΔN variant described here is a cryptic splice isoform that renders the colonic mucosa more susceptible to injury. Our previous studies showed that the N-terminal domain is required for ZBP-89 to mediate

butyrate-dependent activation of $p21^{waf1}$ by cooperating with p300 (2). This result demonstrated that the N terminal domain is functionally important *in vitro*. Generating a mouse model in which only the truncated forms was expressed revealed that loss of the p300-interacting domain results in delayed growth and a shortened lifespan. Although the overall effect on the health of the organism was impressive, an initial survey of several organs did not reveal obvious abnormalities, with the exception of the colon, which appeared to exhibit slightly more inflammatory infiltrates than the wild-type mice. The propensity of the ZBP-$89^{\Delta N}$ expressing mice to develop colitis was uncovered when the animals were challenged with DSS. Therefore, we concluded from these studies that expression of ZBP-89^{FL} tents to protect against colitis. The gene dosage dependence of colitis susceptibility in ZBP-$89^{\Delta N}$ mice suggested that over-expression of ZBP-$89^{\Delta N}$ relative to ZBP-89^{FL} predisposed the mice to inflammation. Similar antagonistic interactions have been reported between FL and ΔN isoforms within the p53 gene family, including ΔNp63 and ΔNp73 (7, 21, 30).

To better understand how the two forms affect downstream targets that participate in the colitis phenotype, we took advantage of the information documenting the role of HSP72 in stress-induced colitis (26). In that study, the authors showed that butyrate treatment ameliorates DSS-induced colitis in rats, an effect associated with suppression of inducible HSP70 (HSP72) (26). When we examined our microarray for downstream targets of ZBP-89, we found that HSP72 expression was suppressed by high levels of ZBP-89 and stimulated if ZBP-89 levels were reduced (Bai and Merchant unpublished). Thus differential regulation of

HSP72 was a reasonable target to consider as an explanation for why loss of ZBP-89^{FL} contributes to colitis. What was not known was whether there was a difference in the ability of the two isoforms to regulate HSP72, a known marker of colitis in humans (13). Therefore, the results that the ZBP-89 $^{\Delta N}$ isoform could not suppress HSP72 suggested that this was at least one mechanism by which its exclusive expression could contribute to inflammation. The differential capacities of the ZBP-89^{-N} and ZBP-89^{FL} isoforms to regulate HSP72 expression in colitis are similar to the opposing actions of p63 isoforms ΔNp63α and TAp63γ in regulating expression of inducible HSP70 (HSP72) in head and neck cancers (28). The latter study showed that the ΔNp63α isoform lacked an HSP70 repression domain located in the amino-terminus of TAp63γ. The ΔNp63α isoform and HSP70 were coordinately over-expressed in cancer, suggesting that activation of HSP70 expression is partly responsible for the oncogenic potential of ΔNp63α (28). This point may be relevant to understanding our prior finding that ZBP-89 is over-expressed in gastric cancer (23).

The ZBP-89$^{\Delta N}$ knock-in model reported here, along with an earlier Zfp148+/- model, help to elaborate how individual protein domains mediate the *in vivo* functions of ZBP-89 (22). Mice heterozygous for a null ZBP-89 allele demonstrate complete failure of male germ cell development and are defective in p53-dependent embryogenesis (22). This finding suggests that the ability of ZBP-89 to interact with p53 is exquisitely sensitive to gene dosage. In contrast, homozygous ZBP-89$^{\Delta N}$ expression is compatible with embryonic and postnatal survival, albeit at reduced levels. We previously showed that the DNA-binding, zinc finger region of ZBP-89

mediates its interaction with p53, and this domain is retained in the ZBP-89$^{\Delta N}$ isoform (22). Therefore, the amino-terminal domain, which includes the p300 interaction domain, is dispensable for p53-dependent embryonic development and postnatal survival, but is essential for normal gastrointestinal function.

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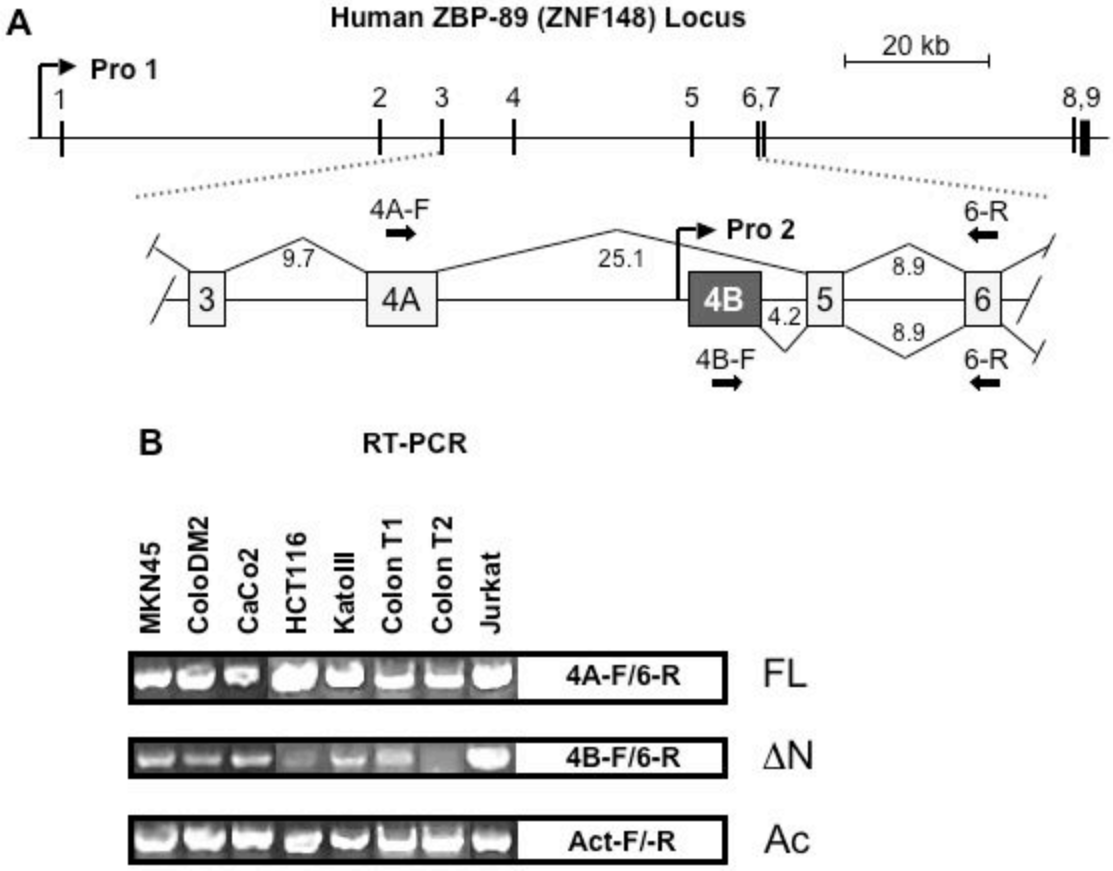

Figure A-1. Identification of a novel exon within the human ZBP-89 (ZNF148) locus. **A)** The human ZBP-89 (ZNF148) locus spans 142 kb (upper panel) and encompasses 3 untranslated (1-3) and 6 coding (4-9) exons. A potential alternative exon 4B was identified at a position 4.2 kb upstream of exon 5. Arrows indicate the locations of forward (F) and reverse (R) RT-PCR primers used for semi-quantitative expression analysis of FL ZBP-89 and the exon 4B variant. Selected intron sizes (kb) are indicated. **B)** RT-PCR analysis of full-length (FL) and variant (DN) ZBP-89 mRNA expression, using actin (Ac) as control. The cDNAs were generated with cultured cell lines using primers 4A-F/6-R (~ 350 bp product); primers 4B-F/6-R (~ 250 bp product); and (Ac), Actin control primers (400 bp product). Whole cell RNA was isolated from MKN 45 and Kato III, human gastric cell lines; and ColoDM2, CaCo 2, HCT116, human colon cell lines as well as two primary colon adenocarcinomas. Whole cell RNA from Jurkat T-cell leukemia cells, where ZBP-89 expression is high, was used as a positive control. Full-length message is expressed from promoter 1 (Pro 1) and variant (DN) ZBP-89 mRNA is expressed from promoter 2 (Pro 2).

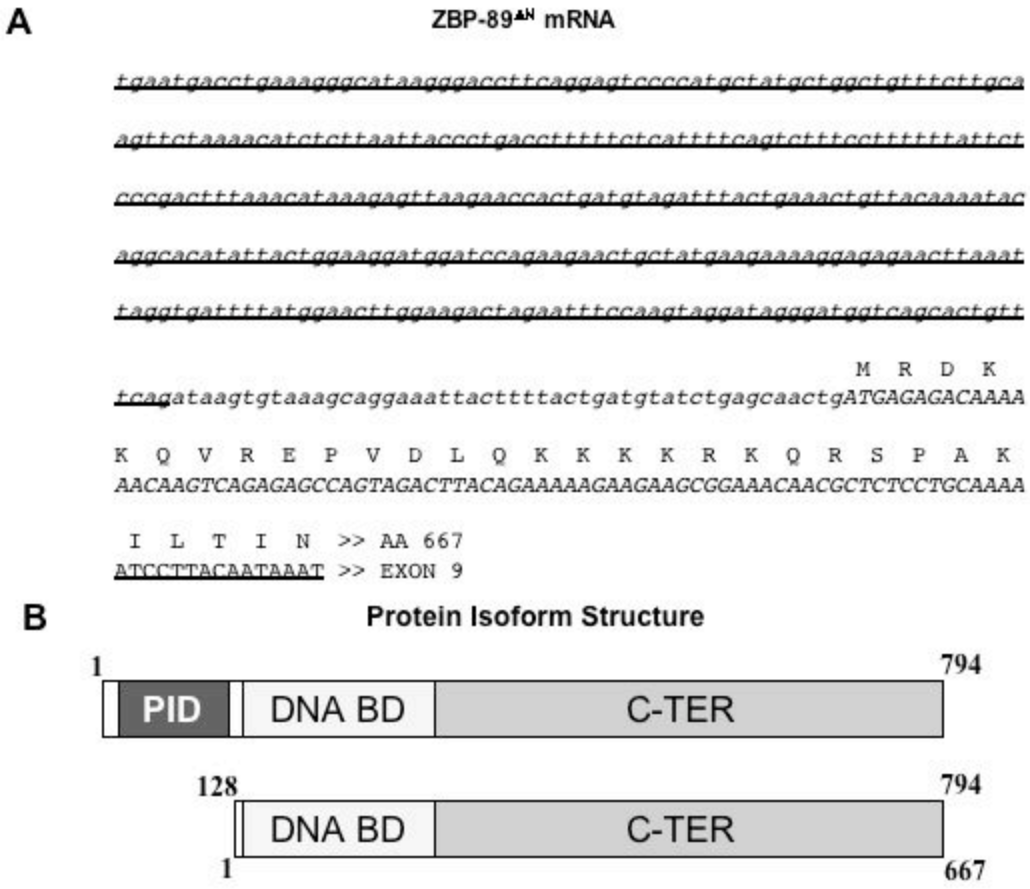

Figure A-2. Variant ZBP-89 transcript encodes a truncated protein, ZBP-89$^{\Delta N}$. **A)** The 5'- end of exon 4B-variant cDNA, encoding ZBP-89$^{\Delta N}$, is shown. Underlined italics, exon 4B-encoded; lower case italics, exon 5 untranslated (frameshift relative to ZBP-89^{FL} cDNA); upper case italics, exon 5 coding sequence commencing at M128 relative to FL protein; upper case underlined, beginning of exon 6-encoded. The remainders of the cDNA and amino acid sequences are identical to the corresponding segments of FL cDNA and protein. **B)** Comparison of FL (upper) and DN (lower) protein isoforms. The amino terminus of ZBP-89^{FL} includes an acidic and p300-interaction domain (PID), which is absent in ZBP-89$^{\Delta N}$. The ZBP-89$^{\Delta N}$ isoform retains the DNA binding and C-terminal domains.

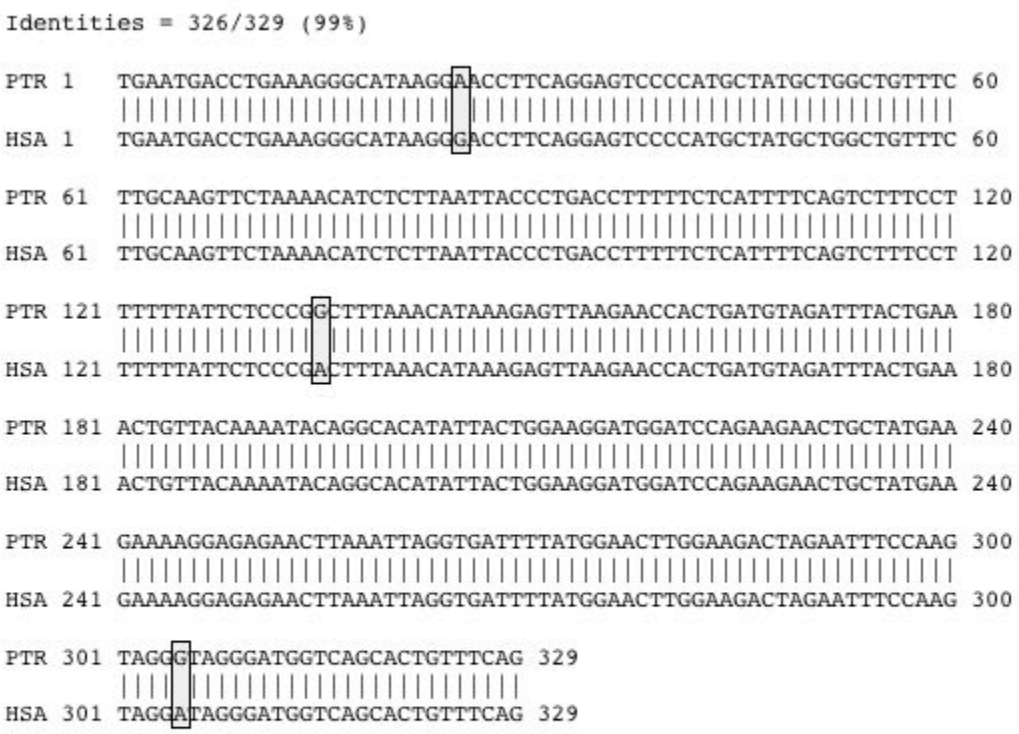

Figure A-3. Identification of ZNF148 exon 4B homology in *Pan troglodytes*. Comparison of *Pan troglodytes* (PTR) DNA to *Homo sapiens* (HSA) human exon 4B genomic sequence (GenBank entry DQ090088) reveals 99% sequence identity. Gray boxes highlight three mismatches.

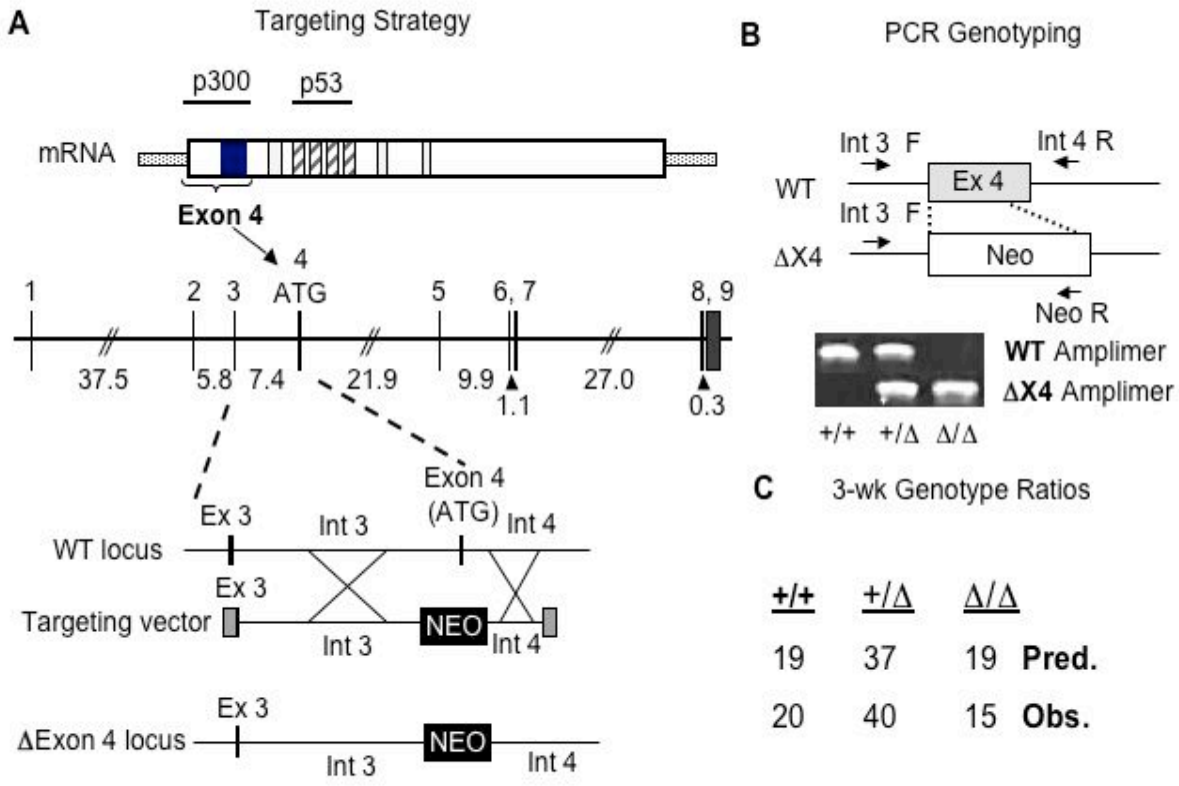

Figure A-4. Targeting ZBP-89 exon 4 in mice. **A)** Replacement of exon 4 with a Neomycin resistance cassette (NEO) by homologous recombination in ES cells. Top panel: bracket shows location of features encoded by exon 4, including initiation codon, acidic domain (black rectangle) and p300 interaction domain. Gray, basic domains; diagonal stripes, zinc fingers; stippled, untranslated. Middle panel; Zfp148 genomic locus. Intron sizes (kb) are shown. Lower panel; targeting strategy. **B)** Tail biopsy DNA genotyping. WT (Int 3 F/Int 4 R) and _exon4 (Int 3 F/Neo R) amplimers. **C)** Near Mendelian inheritance of ΔExon-4 alleles. The differences between predicted and observed values were not significant.

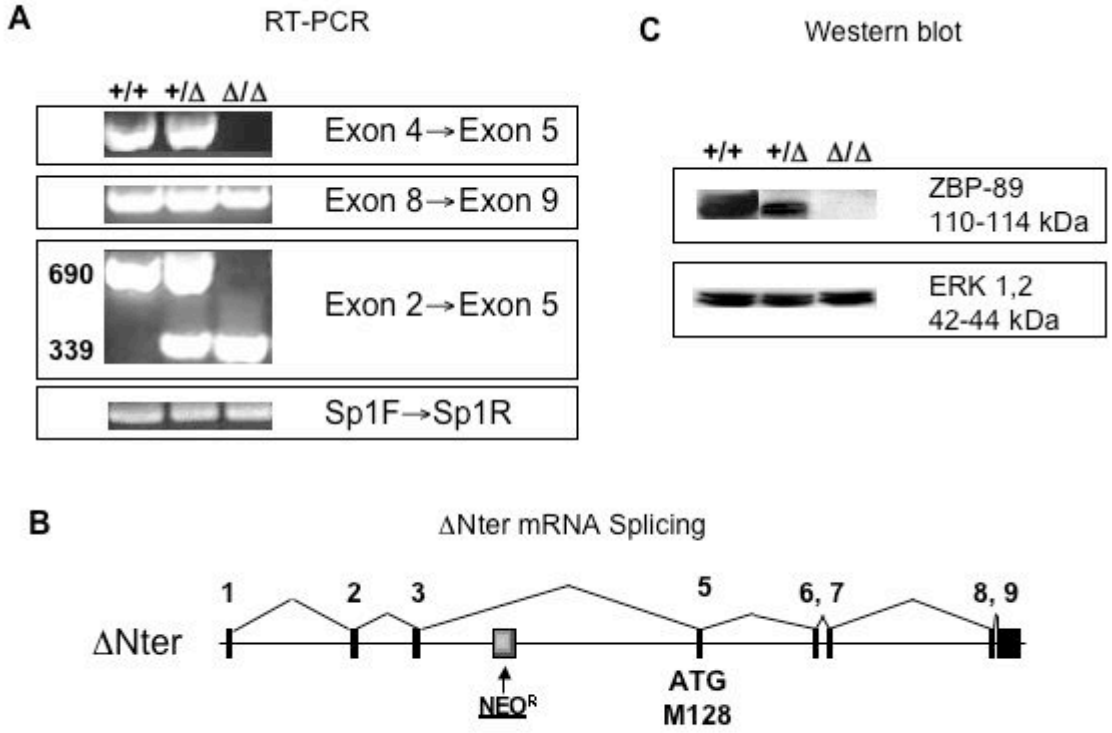

Figure A-5. ΔExon4 locus encodes mouse ZBP-89$^{\Delta N}$. **A)** RT-PCR analysis was used to determine the variant mRNA structure in recombinant mice. Spleen whole-cell RNA was reverse-transcribed and amplified with primers within the indicated exons. RT-PCR of another ubiquitously expressed zinc finger transcription factor, Sp1, was used for comparison and showed that the RNA samples were of uniform quality. Control experiments showed that PCR products were RT-dependent (data not shown), indicating that they were indeed derived from mRNA rather than possibly resulting from amplification of ZBP-89 related processed pseudogene sequences, such as ΨBERF1 (1). **B)** DNA sequencing of recombinant cDNA showed that deletion of exon 4 resulted in direct splicing of exon 3 to exon 5, excluding the Neo cassette. The resulting reading frameshift predicts an alternative initiation codon corresponding to M128 of FL protein, similar to the naturally occurring human ZBP-89$^{\Delta N}$ variant. **C)** Immunoblot analysis: Whole-cell spleen protein extracts from homozygous Δexon4 (Δ/Δ) and control mice (+/+, +/Δ) incubated with a polyvalent ZBP-89 antiserum, raised with an antigen encompassing amino acid residues 1-521. Although the ZBP-89^{FL} protein is absent from Δ/Δ mice, this serum fails to detect a truncated protein, perhaps due to loss or inaccessibility of the relevant epitope.

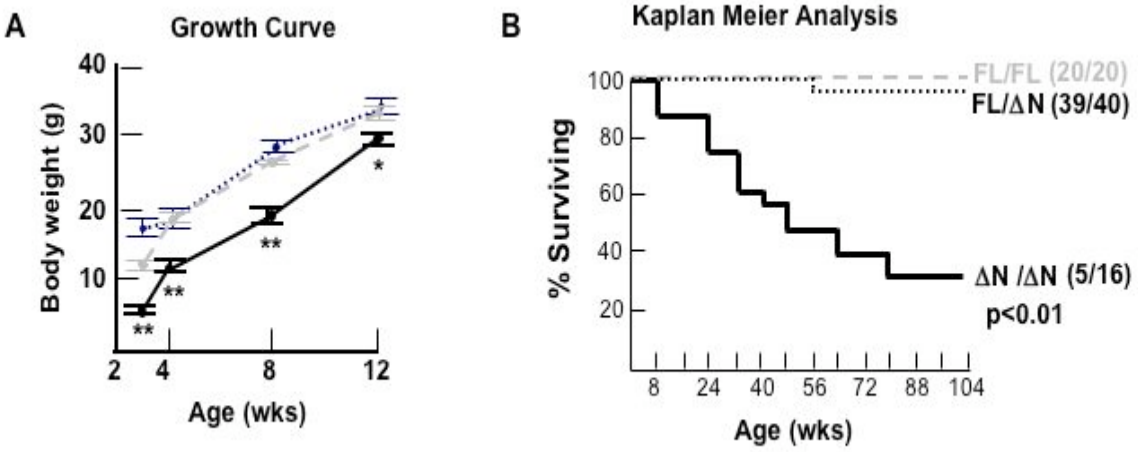

Figure A-6. Growth delay and decreased survival in _Nter mice. **A)** Growth curve for male ZBP-89$^{FL/FL}$ (gray dashed line, n=20), ZBP-89$^{FL/\Delta N}$ (black dotted line, n=36), and ZBP-89$^{\Delta N/\Delta N}$ (black solid line, n=16) offspring. A similar pattern was seen with female mice (not shown). ** p<0.01; * p<0.05. A trend toward lower body weights in ZBP-89$^{\Delta N/\Delta N}$ persisted after 12 weeks, but was no longer statistically significant (not shown). **B)** Kaplan-Meier analysis of survival interval to the onset of morbidity or death.

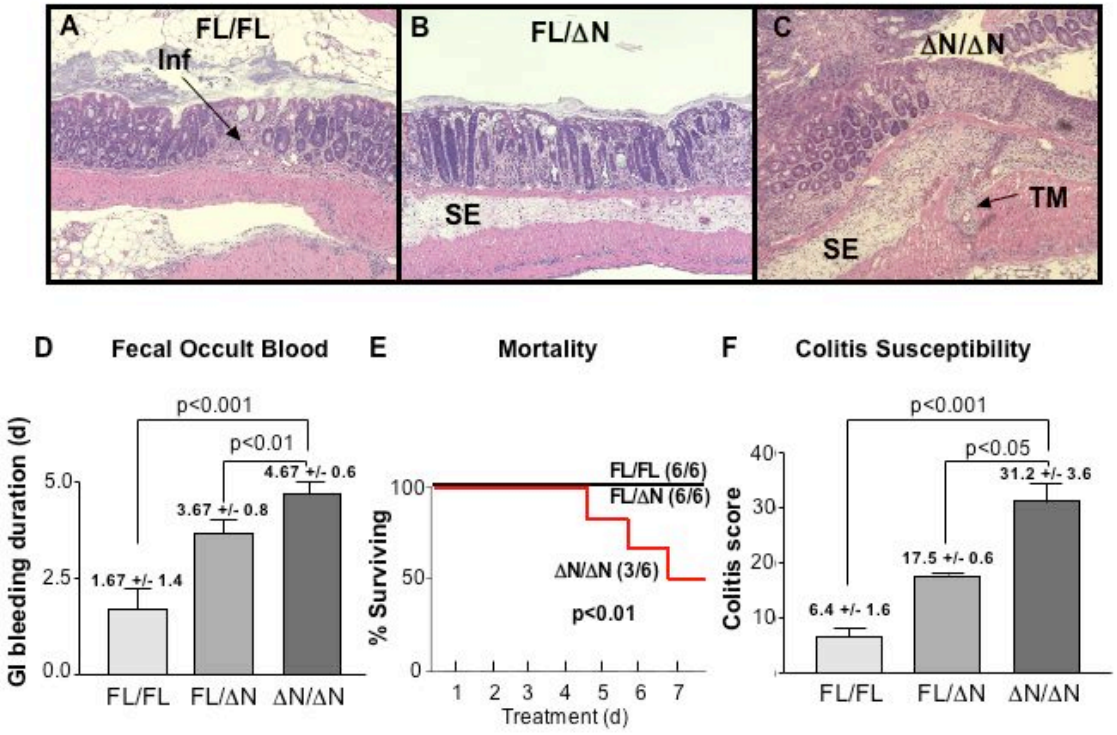

Figure A-7. Increased susceptibility to DSS colitis in ZBP-89$^{\Delta N}$ mice. **A-C)** Representative H&E stains of normal **(A)**, heterozygous **(B)** and ZBP-89$^{\Delta N/\Delta N}$ **(C)** mice. Inf = infiltrating lymphocytes; SE = submucosal edema; TM = transmural inflammation. **D)** ΔNter expression accelerates onset of gastrointestinal bleeding, measured by guiac fecal occult blood screening. **E)** Mortality during DSS treatment: 50% of ZBP-89$^{\Delta N}$/ZBP-89$^{\Delta N}$ mice die during 4% DSS treatment; all ZBP-89^{FL}/ZBP-89^{FL} and ZBP-89^{FL}/ZBP-89$^{\Delta N}$ mice survived (6 in each group). **F)** Colitis index scoring of DSS-treated mice, as described in Materials and Methods; 6 mice in each genotype cohort.

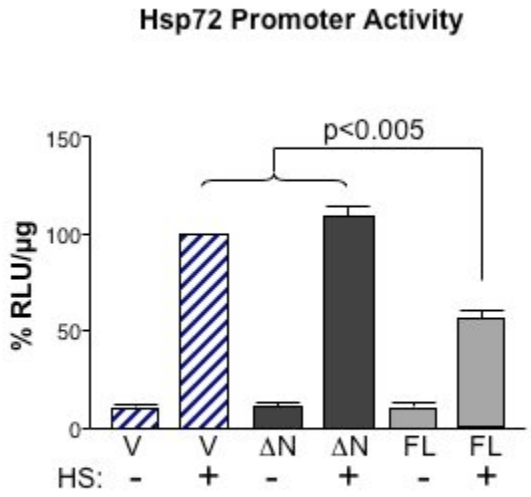

Figure A-8. ZBP-89$^{\Delta N}$ lacks the ability of ZBP-89^{FL} to repress HSP72 expression. ZBP-89^{FL} and ZBP-89$^{\Delta N}$ expression constructs were co-transfected with the HSP72 promoter into HT-29 colon cancer cells. Heat shock was applied for 90 min at 43 C. Values are normalized to cells transfected with empty vector after receiving heat shock. Results are calculated from 4 independent assays.

References

1. Bai, L., and J. L. Merchant. 2000. Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. J Biol Chem 275:30725-33.

2. Bai, L., and J. L. Merchant. 2001. ZBP-89 promotes growth arrest through stabilization of p53. Mol Cell Biol 21:4670-83.

3. Brett, D., H. Pospisil, J. Valcarcel, J. Reich, and P. Bork. 2002. Alternative splicing and genome complexity. Nat Genet 30:29-30.

4. Brinkman, B. M. 2004. Splice variants as cancer biomarkers. Clin Biochem 37:584-94.

5. Cartegni, L., S. L. Chew, and A. R. Krainer. 2002. Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet 3:285-98.

6. Feo, S., V. Antona, G. Cammarata, F. Cavaleri, R. Passantino, P. Rubino, and A. Giallongo. 2001. Conserved structure and promoter sequence similarity in the mouse and human genes encoding the zinc finger factor BERF-1/BFCOL1/ZBP-89. Biochem Biophys Res Commun 283:209-18.

7. Hashimoto, Y., C. Zhang, J. Kawauchi, I. Imoto, M. T. Adachi, J. Inazawa, T. Amagasa, T. Hai, and S. Kitajima. 2002. An alternatively spliced isoform of transcriptional repressor ATF3 and its induction by stress stimuli. Nucleic Acids Res 30:2398-406.

8. Hui, L., X. Zhang, X. Wu, Z. Lin, Q. Wang, Y. Li, and G. Hu. 2004. Identification of alternatively spliced mRNA variants related to cancers by genome-wide ESTs alignment. Oncogene 23:3013-23.

9. Kalnina, Z., P. Zayakin, K. Silina, and A. Line. 2005. Alterations of pre-mRNA splicing in cancer. Genes Chromosomes Cancer 42:342-57.

10. Kulp, D., D. Haussler, M. G. Reese, and F. H. Eeckman. 1996. A generalized hidden Markov model for the recognition of human genes in DNA. Proc Int Conf Intell Syst Mol Biol 4:134-42.

11. Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J. P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J. C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R. H. Waterston, R. K. Wilson, L. W. Hillier, J. D. McPherson, M. A. Marra, E. R. Mardis, L. A. Fulton, A. T. Chinwalla, K. H. Pepin, W. R. Gish, S. L. Chissoe, M. C. Wendl, K. D. Delehaunty, T. L. Miner, A. Delehaunty, J. B. Kramer, L. L. Cook, R. S. Fulton, D. L. Johnson, P. J. Minx, S. W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J. F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, et al. 2001. Initial sequencing and analysis of the human genome. Nature 409:860-921.

12. Law, D. J., S. A. Tarle, and J. L. Merchant. 1998. The human ZBP-89 homolog, located at chromosome 3q21, represses gastrin gene expression. Mamm Genome 9:165-7.

13. Ludwig, D., M. Stahl, E. T. Ibrahim, B. E. Wenzel, D. Drabicki, A. Wecke, K. Fellermann, and E. F. Stange. 1999. Enhanced intestinal expression of heat shock protein 70 in patients with inflammatory bowel diseases. Dig Dis Sci 44:1440-7.

14. Mahler, M., I. J. Bristol, E. H. Leiter, A. E. Workman, E. H. Birkenmeier, C. O. Elson, and J. P. Sundberg. 1998. Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. Am J Physiol 274:G544-51.

15. McGinnis, S., and T. L. Madden. 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res 32:W20-5.

16. Merchant, J. L., G. R. Iyer, B. R. Taylor, J. R. Kitchen, E. R. Mortensen, Z. Wang, R. J. Flintoft, J. B. Michel, and R. Bassel-Duby. 1996. ZBP-89, a Kruppel-like zinc finger protein, inhibits epidermal growth factor induction of the gastrin promoter. Mol Cell Biol 16:6644-53.

17. Moreau, N. M., M. M. Champ, S. M. Goupry, B. J. Le Bizec, M. Krempf, P. G. Nguyen, H. J. Dumon, and L. J. Martin. 2004. Resistant starch modulates in vivo colonic butyrate uptake and its oxidation in rats with dextran sulfate sodium-induced colitis. J Nutr 134:493-500.

18. Myers, K. J., S. Murthy, A. Flanigan, D. R. Witchell, M. Butler, S. Murray, A. Siwkowski, D. Goodfellow, K. Madsen, and B. Baker. 2003. Antisense oligonucleotide blockade of tumor necrosis factor-alpha in two murine models of colitis. J Pharmacol Exp Ther 304:411-24.

19. Rachmilewitz, D., F. Karmeli, K. Takabayashi, T. Hayashi, L. Leider-Trejo, J. Lee, L. M. Leoni, and E. Raz. 2002. Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. Gastroenterology 122:1428-41.

20. Reese, M. G., F. H. Eeckman, D. Kulp, and D. Haussler. 1997. Improved splice site detection in Genie. J Comput Biol 4:311-23.

21. Stiewe, T., S. Zimmermann, A. Frilling, H. Esche, and B. M. Putzer. 2002. Transactivation-deficient DeltaTA-p73 acts as an oncogene. Cancer Res 62:3598-602.

22. Takeuchi, A., Y. Mishina, O. Miyaishi, E. Kojima, T. Hasegawa, and K. Isobe. 2003. Heterozygosity with respect to Zfp148 causes complete loss of fetal germ cells during mouse embryogenesis. Nat Genet 33:172-6.

23. Taniuchi, T., E. R. Mortensen, A. Ferguson, J. Greenson, and J. L. Merchant. 1997. Overexpression of ZBP-89, a zinc finger DNA binding protein, in gastric cancer. Biochem Biophys Res Commun 233:154-60.

24. Tybulewicz, V. L., C. E. Crawford, P. K. Jackson, R. T. Bronson, and R. C. Mulligan. 1991. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell 65:1153-63.

25. Venables, J. P. 2004. Aberrant and alternative splicing in cancer. Cancer Res 64:7647-54.

26. Venkatraman, A., B. S. Ramakrishna, R. V. Shaji, N. S. Kumar, A. Pulimood, and S. Patra. 2003. Amelioration of dextran sulfate colitis by butyrate: role of heat shock protein 70 and NF-kappaB. Am J Physiol Gastrointest Liver Physiol 285:G177-84.

27. Woodley, L., and J. Valcarcel. 2002. Regulation of alternative pre-mRNA splicing. Brief Funct Genomic Proteomic 1:266-77.

28. Wu, G., M. Osada, Z. Guo, A. Fomenkov, S. Begum, M. Zhao, S. Upadhyay, M. Xing, F. Wu, C. Moon, W. H. Westra, W. M. Koch, R. Mantovani, J. A. Califano, E. Ratovitski, D. Sidransky, and B. Trink. 2005. DeltaNp63alpha up-regulates the Hsp70 gene in human cancer. Cancer Res 65:758-66.

29. Xu, Q., and C. Lee. 2003. Discovery of novel splice forms and functional analysis of cancer-specific alternative splicing in human expressed sequences. Nucleic Acids Res 31:5635-43.

30. Zaika, A. I., N. Slade, S. H. Erster, C. Sansome, T. W. Joseph, M. Pearl, E. Chalas, and U. M. Moll. 2002. DeltaNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors. J Exp Med 196:765-80.

31. Zavolan, M., S. Kondo, C. Schonbach, J. Adachi, D. A. Hume, Y. Hayashizaki, and T. Gaasterland. 2003. Impact of alternative initiation, splicing, and termination on the diversity of the mRNA transcripts encoded by the mouse transcriptome. Genome Res 13:1290-300.